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CONTROLLED DELIVERY OF ANTIBACTERIALS USING POLYCAPROLACTONE MATRICES FOR THE INTRAVAGINAL TREATMENT OF SEXUALLY TRANSMITTED INFECTIONS

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Abstract

Around 500 million cases of four of the major curable sexually transmitted infections (STIs), *Chlamydia*, gonorrhoea, syphilis and trichomoniasis, were recorded worldwide in 2008, an increase of 11% from 2005, confirming the need for improved prevention and treatment strategies. The chances of acquiring these infections are 8 times greater in women than men because of biological, social and cultural factors. Furthermore, the presence of these infections increases the chances of both acquisition and transmission of HIV/AIDS by causing genital ulcers and inflammation in the vagina.

The human vagina is considered to be a novel, non-invasive and safe route for drug delivery because of its rich blood supply, large surface area and low enzymatic activity. There are many vaginal preparations available in the market but conventional systems such as creams and gels are criticised because of messiness and leakage, and many vaginal formulations have the disadvantage of daily dosing. Intravaginal rings (IVRs) have potential advantages over other delivery systems, in that they can be used for prolonged periods of time, avoid messiness and sustained drug delivery is possible. Previous research has focussed on the use of IVRs for the prevention of HIV. This thesis describes the development of IVRs for the treatment of vaginal bacterial and fungal infections, including STIs.

The most important component of IVRs is the polymer used in their construction; currently IVRs are composed of silicone and polyethylene vinyl acetate but these require a high processing temperature and are suitable only for delivery of low molecular weight, hydrophobic drugs. In this thesis, the use of polycaprolactone (PCL) as a potential polymer for use in IVRs is investigated because of its perceived advantages over other polymers, such as low processing temperature and potential to deliver a wide range of drug molecules from hydrophobic to hydrophilic, and low-molecular to high molecular weight.

Following a review of the literature in Chapter 1, the delivery of metronidazole using PCL, which could be used for the treatment of bacterial vaginosis, is considered in Chapter 2. Delivery of metronidazole in an IVR could be a better option than the vaginal gel and oral tablet formulations that are currently available because an IVR would provide long term sustained delivery and would be expected to reduce the gastrointestinal side effects associated with oral delivery. PCL matrices loaded with different concentrations of

metronidazole achieved an incorporation efficiency of 40-54%. The matrices were studied using a range of approaches including release into simulated vaginal fluid (SVF), morphological and drug distribution studies, thermal characterization and antibacterial activity against *Gardnerella vaginalis* (one of the main bacteria implicated in bacterial vaginosis). First day burst release occurred due to the presence of drug crystals at the surface of the PCL which was confirmed by scanning electron microscopy and Raman microscopy. Even considering the small decrease in antibacterial activity that was measured during the 14 day test period, drug release on each of the 14 days was greater than the minimum inhibitory concentration (MIC) against *G. vaginalis*. The effect of polyethylene glycol (PEG) addition on drug release and mechanical properties of the PCL was then investigated. Different concentrations of PEG were loaded into PCL matrices along with 10% w/w metronidazole. Increasing the concentration of PEG enhanced both drug loading and the amount of daily drug release, but this was associated with a negative effect on the mechanical properties of polymer and made it more soft and brittle. It was concluded that PCL has potential to be a useful polymer for use in IVR delivery of metronidazole but that there was still capacity to improve drug loading.

Chapter 3 describes experiments with PCL matrices loaded with doxycycline, which can be used for the treatment of gonorrhoea and *Chlamydia*. A slight alteration to the method of production of PCL matrices enabled 100% drug loading to be obtained. Following the same suite of tests described for Chapter 2, it was concluded that these PCL matrices can deliver doxycycline effectively for 14 days, and the concentrations released *in vitro* on each of the 14 days were greater than the minimum inhibitory concentration (MIC) against several pathogens that are sexually transmitted. Additionally, the toxicity of PCL leachates was tested on the vaginal cell line VK2/E6E7 and found to be safe for vaginal delivery. There are currently no commercial vaginal preparations of doxycycline so this study could be useful in introducing a new drug delivery system for doxycycline through the intravaginal route.

In Chapter 4, the ability of PCL to deliver a combination of drugs is investigated. Metronidazole and doxycycline are the combination involved, which are used together for the treatment of pelvic inflammatory disease. PCL matrices loaded with different concentrations of metronidazole and doxycycline were tested for *in vitro* drug release, morphological, thermal and antibacterial testing to investigate whether the combination compromised the effectiveness of the PCL matrix in drug delivery. Excellent drug loading

of both drugs was obtained, and the concentrations released for each of 14 days were associated with a high level of antibacterial activity. This is the first investigation of the vaginal delivery of metronidazole and doxycycline in combination.

The delivery of a macromolecular protein using PCL matrices is investigated in Chapter 5. This study is based on the fact that vaginal vaccination is potentially a more effective means of eliciting a strong localized immune response against HIV, in comparison to conventional intramuscular or intranasal routes of administration, and most of these vaccines are either proteins or peptides in nature. Lactoferrin was used as a model protein for the study because of its reported activity against herpes simplex virus, *Chlamydia trachomatis* and HIV. PCL matrices proved suitable for the delivery of lactoferrin; the integrity of the protein was retained as shown using SDS-PAGE of the protein following loading into PCL and release into SVF.

These findings could provide a breakthrough in the field of vaginal drug delivery for the treatment of STIs and could also reduce the risk of HIV infection. Using PCL matrices in intravaginal rings for vaginal delivery of microbicides as a strategy to treat vaginal infections and STIs is expected to reduce the risks of drug resistance, treatment failure and gastrointestinal adverse effects associated with long-term oral drug delivery, by improving compliance with treatment duration and avoiding systemic adverse drug reactions. Future research should investigate the mechanical properties of PCL, which may limit the preparation of simple IVRs, and consider the use of drug-loaded PCL as inserts within a flexible inert IVR, or covering the drug-loaded PCL IVR with a flexible inert material with delivery windows that allow drug release. Mixing PCL with a second polymer such as polyethylene may also be considered but it would be important not to destroy the usefulness of PCL in terms of its low processing temperature.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Conference abstracts

1. **Meenakshi Pathak**, Mark Turner, Cheryn Palmer, Allan Coombes- Evaluation of polycaprolactone matrices for the intravaginal delivery of metronidazole in the treatment of bacterial vaginosis, Drug delivery Australia (DDA), Sydney, Australia, 2013 (Poster presentation).

2. **Meenakshi Pathak,** Allan G A Coombes, Mark Turner, Cheryn Palmer, Kathryn J. Steadman - Investigation of polycaprolactone matrices containing doxycycline and metronidazole for the treatment of pelvic inflammatory disease, International Pharmaceutical Federation (FIP), Melbourne, Australia, 2014 (Oral presentation).

3. **Meenakshi Pathak,** Mark Turner, Cheryn Palmer, Kathryn J. Steadman - Investigation of polycaprolactone matrices containing doxycycline for the treatment of gonorrhoea, ASMR Post Graduate Student conference, Brisbane, Australia, 2014 (Poster presentatinon).

4. **Meenakshi Pathak,** BoMi Ryu, Mark Turner, Peter Cabot, Kathryn J. Steadman - Investigation of polycaprolactone matrices for the sustained intravaginal delivery of proteins, D4 conference, Otago, New Zealand, 2014 (Oral Presentation).
Abstract selected for the NZCRS student speaker prize.

5. **Meenakshi Pathak,** BoMi Ryu, Mark Turner, Peter Cabot, Kathryn J. Steadman - Investigation of polycaprolactone matrices for the sustained intravaginal delivery of proteins, Australasian Pharmaceutical Science Association (APSA) Conference, Brisbane, Australia, 2014 (Oral Presentation).

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Contributor	Statement of contribution
Meenakshi Pathak (Candidate)	Experimental design 70%, data collection 100% except microbiology 80%, interpretation of data 70%, drafting and writing 100%
Mark Turner	Microbiology experimentation 20% and manuscript revision 20%
Cheryn Palmer	Supervision and manuscript revision 10%
Allan G A Coombes	Experimental design 30%, interpretation of data 30% and manuscript revision 70%

2. **Pathak M**, Coombes AG, Turner MS, Palmer C, Wang D, Steadman KJ. Investigation of polycaprolactone matrices for intravaginal delivery of doxycycline. Journal of Pharmaceutical Sciences 2015 104:4217-4222. Incorporated in Chapter 3.

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Allan G A Coombes	Interpretation of data 10% and manuscript revision 30%
Cheryn Palmer	Supervision and manuscript revision 10%
Mark Turner	Microbiology experimentation 20% and manuscript revision 10%
Dongjie Wang	SEM 50%
Kathryn J Steadman	Interpretation of data 10%, Manuscript revision 50%

Contributions by others to the thesis

A/Prof Peter Cabot and Dr BoMi Ryu (School of Pharmacy, UQ) contributed to setup of the cell culture experiment (Chapter 3), Dr Dongjie Wang (Queensland Alliance for Agriculture and Food Innovation, UQ) helped with Scanning Electron Microscopy (Chapter 3, 4 and 5), and Manasi Jambhrunkar (Australian Institute of Biotechnology and Nanotechnology) conducted the SDS-PAGE (Chapter 5).

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

Vaginal drug delivery, sexually transmitted infections, polycaprolactone, drug delivery systems, intravaginal rings

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Abstract

This review describes the intended use of polycaprolactone (PCL) for the preparation of intravaginal rings (IVRs) that may be used for vaginal delivery of antibacterials in the treatment of curable sexually transmitted infections (STIs). PCL has certain characteristics (biocompatibility, permeability and low processing temperature) that are advantageous when compared to polymers that are currently used in the production of IVRs such as silicone and polyethylene vinyl acetate. Vaginal drug delivery using IVRs has several advantages over other routes of administration because drug delivery can occur over a long period of time from a single ring, first pass metabolism is bypassed, and rings are generally regarded as comfortable and acceptable to use. Curable STIs are a major concern because of their increasing number and their adverse effect on the health. Current treatments, involving creams, gels and pessaries, required daily administration and can be messy, leading to relatively low acceptability. There are several recent review articles published on the topic of IVRs but most of these articles concentrate on the use of rings in HIV/AIDS prevention or as contraceptives. Little attention has previously been given to the use of IVRs for the delivery of antibacterials for the treatment of curable STIs. This review highlights the potential use of IVRs for the treatment of curable STIs, explains the advantages of these delivery systems over other solid and semi-solid vaginal preparations, and considers the potential benefits of PCL as a polymer for vaginal drug delivery.

2. Introduction

Most treatable vaginal infections are sexually transmitted (STIs, Table 1), but while these are the major focus of this review some consideration is given to certain infections that are not caused by sexual activity but are important in vaginal infections such as bacterial vaginosis and candidiasis. There are several recent review articles published about IVRs but the articles concentrate on their use as a prevention strategy for HIV/AIDS or their use as contraceptives. STIs are of considerable concern globally due to their adverse effect on quality of life, pregnancy-related complications, burden to the economy and the fact that they are increasing in prevalence globally[1]. Additionally, the presence of many untreated STIs and an unhealthy vaginal system increases the risk of both acquisition and transmission of HIV due to i) genital ulceration which facilitates entry of HIV through breaks in the vaginal epithelia[2] ii) inflammation caused by the STIs which attracts various types of cells such as CD4+ and T-cells that are primary targets of HIV[2] and iii) increase in vaginal pH which reduces the vaginal defence against pathogens[3]. The most common

symptoms associated with STIs are vaginal infections, vaginal discharge and cervicitis (Figure 1) which can cause disturbance in vaginal environment and can make it susceptible to other infections.

Table 1: Common causes of sexually transmitted and vaginal infections[4-6]

STI	Cause
Bacterial Infections	
Gonorrhea	*Neisseria gonorrhoeae*
Chlamydia	*Chlamydia trachomatis*
Chancroid	*Haemophilus ducreyi*
Donovanosis	*Klebsiella granulomatis*
Mycoplasma	*Mycoplasma genitalium*
Syphilis	*Treponema pallidum*
Viral Infections	
Acquired immunodeficiency syndrome	*Human immunodeficiency virus*
Genital herpes	*Herpes simplex* 1 & 2
Genital warts	*Human papillomavirus*
Protozoa	
Trichomoniasis	*Trichomonas vaginalis*
Fungal	
Candidiasis	*Candida albicans*
Parasitic Infections	
Scabies	*Sarcoptes scabiei*
Pubic lice	*Phthirus pubis*
Other vaginal infections	
Bacterial vaginosis	Polymicrobial (*Gardnerella vaginalis, Prevotella* spp., *Mobiluncus* spp., *Ureaplasma urealyticum and Mycoplasma homonis)*

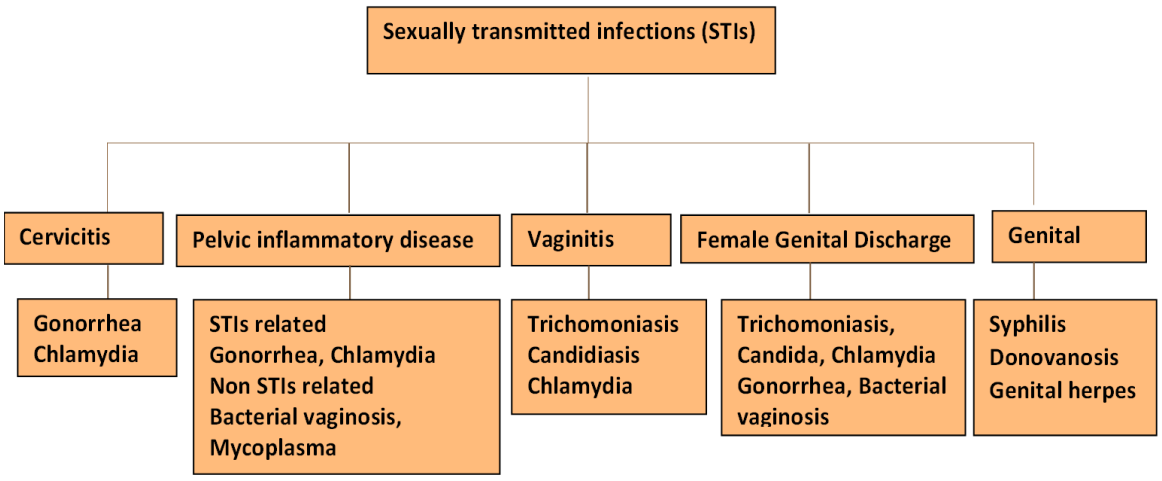

Figure 1: Schematic representation of the most important symptoms and causes of sexually transmitted infections (STIs) and vaginal infections

In 2012, a WHO report indicated that four major curable STIs had increased in number by 11.3% worldwide within 3 years[1]. Although this doesn't incorporate the concomitant increase in global population, as most of the diseases can be asymptomatic so the numbers may be an underrepresentation.

The chances of male to female transmission of STIs is 8 times higher than the female to male transmission and that may be due to social, cultural and biological factors[4]. STIs and their reoccurrences are preventable and there are many health promotion activities trying to prevent these infections in a cost effective way. However, the long duration of treatment and side effects associated with the prescribed drugs, usually given by the oral or parenteral route, make the therapy uncomfortable for the patients. Consequently, non-adherence to the treatment regimen is a major problem, which leads to repetitive episodes of STIs and development of resistance to many drugs[5]. WHO considers non-adherence to be an alarming factor and addresses the need for adherence to therapy[6].

3. The vagina as a route for drug delivery

The vagina is considered to be a favourable site for both local and systemic delivery of drugs because of its large surface area and permeability to a large variety of compounds such as prostaglandins and steroids[4]. The vaginal epithelium has relatively few blood vessels so topical drug delivery for local action can be effective. For example the topical intravaginal delivery of microbicides results in high concentrations in the genital compartment rather than than systemic circulation[7]. It may also be a favourable site for the administration of peptides and proteins targeting the mucosal immune system[8]. Another option is as a site for systemic delivery; as the GI lumen and the liver are the primary sites of elimination for many compounds, vaginal administration may be preferable to the oral route due to avoidance of both gastrointestinal absorption and the hepatic first pass effect. Orally delivered drugs may cause side effects such as vomiting, and drug-drug or drug-food interference can also affect drug absorption[9]. Consequently, vaginal administration can be more convenient in terms of allowing more prolonged dosing, lower exposure to drugs and continuous drug delivery[10]. Other advantages of intravaginal drug delivery systems are rapid drug absorption and quick onset of action, and self-administration.

3.1 Vagina anatomy and physiology

The human vagina (Figure 2) extends from the uterus, and is situated behind the bladder and in front of the rectum; it is directed upward and backward, its axis forming an angle of

over 90° with the uterus, opening forward. It may be described as a fibromuscular tube approximately 7-10 cm in length. The position and size of the vagina play an important role in designing a vaginal drug delivery product. For example, vaginal gels have the disadvantage of leakage particularly due to the angle of the vagina, and the dimension of the vagina must be considered when determining the dimensions of intravaginal rings (IVRs).

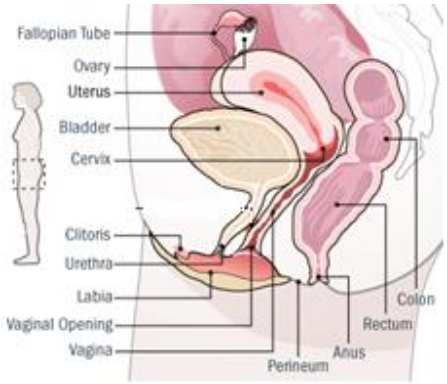

Figure 2: Anatomy of the human vagina[11]

Vaginal histology is composed of four distinct layers (Figure 3):

1. The non-secretory stratified squamous epithelium forms the superficial layer. This layer can be divided into different classes according to the stage of maturation: basal, parabasal, intermediate and superficial. During maturation, cells move from the basal layer to the superficial layer, becoming flatter, with small nuclei and a larger cell volume. The thickness of cervical squamous epithelium varies from (0.2 to 0.5 mm) and depends on the age[12].

2. The lamina propria, or tunica, made of collagen and elastin, contains vascular and lymphatic channels.

3. The muscle layer comprises smooth muscle fibres running in both circular and longitudinal directions and contains a rich blood supply.

4. The final layer called tunica adventitia consists of areolar connective tissue and a large plexus of blood vessels.

The elasticity of the vagina is due to the presence of smooth elastic fibres in the muscular layer and the loose connective tissue of the tunica adventicia[13].

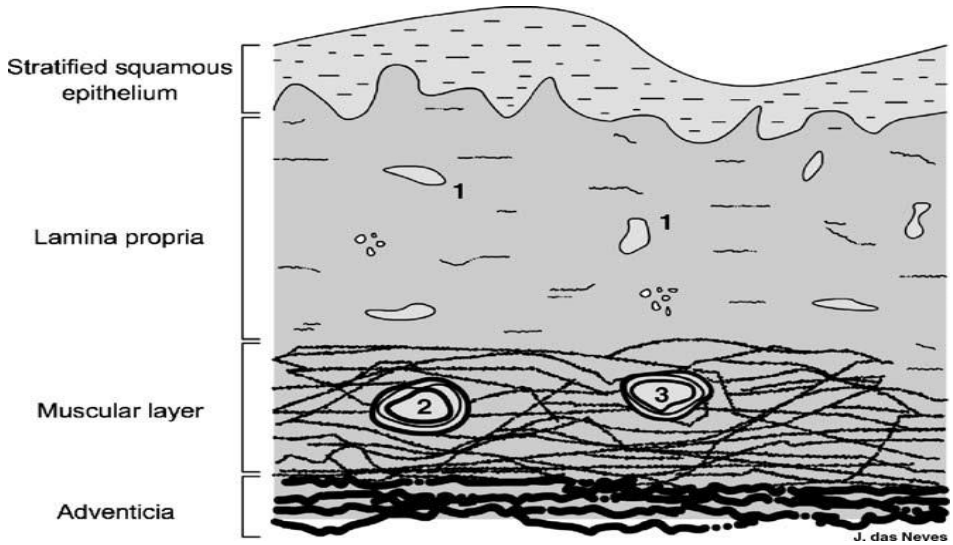

Figure 3: Histology of the vagina. 1: capillary vessels; 2: artery; 3: vein[14]

The surface of the vagina is composed of numerous folds or rugae. The rugae provide support, distensibility, and an increased surface area of the vaginal wall. Blood is supplied to the vagina by the networks of blood vessels extending from the internal iliac artery, uterine, middle rectal and internal pudendal arteries[12]. The lower part of vagina receives its nerve supply from the pudendal nerve and from the inferior hypogastric and uterovaginal plexus. The upper part of the vagina has much fewer sensory fibres making it relatively insensitive, which is the reason women can't feel the presence of IVRs, tampons and pessaries inside the vagina[15].

3.2 Vaginal defence mechanisms

3.2.1 Vaginal epithelium

The superficial layer is the stratified squamous epithelium (Figure 3), which is the primary vaginal defence mechanism as it sheds continuously, making it difficult for microorganisms to invade the basement tunica adventitia[16]. Additionally, vaginal fluids spread over the wall of the vaginal epithelium to form a protective layer that serves as a barrier for the invading bacteria to protect the vagina from infection.

3.2.2 Bacterial flora

The microorganisms present depends upon the physiological conditions of the vagina, which further depend on other factors such as age, time of the menstrual cycle, pregnancy, menopause, infection and douching practices. The vaginal microflora consists of both gram-positive and gram-negative species for both cocci and bacilli[17]. Lactobacilli, which are gram-positive bacteria, are beneficial for vaginal health because they compete

with exogenous microbes for nutrients. Conversion of glycogen present in exfoliated cells into lactic acid by these microorganisms helps to maintain the pH of the vagina[18]. Lactobacilli may or may not produce hydrogen peroxide H_2O_2 depending on the strain. H_2O_2 is toxic to other microorganisms that produce little or no H_2O_2 scavenging enzyme such as catalase. An absence of H_2O_2 producing lactobacilli in the normal vaginal flora can result in bacterial vaginosis or an overgrowth of catalase–negative organisms[18].

3.2.3 Immune cells

Protective immunity against pathogens is provided by both cellular and humoral systems. Langerhans cells are present in the lumen of the vagina and provide local immunity[19]. These cells can pass antigens to the dendritic cells that migrate to the lymph nodes, where they activate B and CD4+ T cells. These activated B lymphocytes return to the subepithelium and turn into IgA secreting cells and, along with IgG, IgM and IgA antibodies present in the cervical mucus, act as a defence mechanism for the vagina[20].

4. Vaginal drug absorption

There are some drug properties that affect their absorption such as molecular weight, oil/water partition coefficient and ionic character[21]. As the wall of the vagina is made up of epithelial tissues, absorption through the vagina is similar to that of other epithelial tissues and can be best explained by the fluid mosaic model as a hydrophobic lipid layer interspersed with aqueous pores[22]. The transport mechanism of most vaginal absorbed substances is simple diffusion. Lipophilic substances are absorbed through the intracellular pathway, whereas hydrophilic substances are absorbed through the intercellular pathway or across aqueous pores present in the vaginal mucosa[23].

4.1 Factors affecting vaginal drug absorption

There are a number of factors worth considering in terms of drug absorption from the vagina. Firstly, due to the anatomical position of the vagina some liquid and semisolid preparations may be expelled under the influence of gravity, resulting in leakage. Secondly, the vaginal epithelium undergoes cyclic changes, under the influence of hormones such as oestrogen, progesterone, luteinising hormone, follicle stimulating hormone and the different phases of the menstrual cycle[24]. In the late follicular phase, the epithelium thickness is increased due to the proliferation of the cells in the basal layer, stimulated by oestrogen and at the same time the number of intercellular junctions also increases with narrow intercellular channels. In the luteal phase, there is desquamation of

the superficial epithelial layer so that epithelium becomes loose with the widening of the intercellular channels. In this case, there is a possibility of absorption of high molecular weight hydrophilic drugs[25]. Thirdly, vaginal pH also influences the drug absorption. Since many drugs are weak electrolytes, the pH may change their degree of ionization and, therefore, affect absorption. For example *in vitro* studies have shown that the release of prostaglandinE2 from vaginal preparations may vary depending on the pH of the media[26], and diffusion of nonoxynol 9 into cervical mucus is pH dependent[13].

4.2 Vaginal systemic drug delivery

There are a number of examples of the study of drug administration via the vagina for systemic delivery:

- The steroids estradiol, estrone, progesterone, medroxy progesterone acetate, norgestrel, norethisterone and testosterone can all be successfully absorbed through the vaginal wall, as shown by measurement of systemic levels of these drugs in humans. Steroids with greater lipophilicity (such as progesterone and estrone) show more rapid absorption than those with less lipophilicity (such as hydrocortisone and testosterone)[10]. A lactose based progesterone vaginal tablet has been designed to deliver biologically effective amounts of progesterone for up to 48 h. When administered into the vagina these tablets form a milky suspension and provide advantages for the treatment of menstrual irregularities, functional uterine bleeding, luteal phase defects, premenstrual tension, infertility and osteoporosis[27].

- Intravaginal delivery of indomethacin has been investigated for the treatment of preterm labour. Indomethacin, a potent inhibitor of prostaglandin synthesis and has been used effectively as a labour inhibiting agent for 48 h in pregnancies of less than 32 weeks. Indomethacin has been proven to be more effective when used intra-vaginally as compared to an intrarectal plus oral regimen, whereby delivery of the baby was delayed by more than 7 days in 78% of women who received the drug intravaginally compared with 43% who received the same dose rectal-orally[28].

- Intravaginal metronidazole administration has been investigated for systemic delivery. A solution at 10 mg/kg administered either orally or intra-vaginally to rats indicated that absorption of metronidazole from the vagina was rapid, with only 4% of the dose remaining in the vagina after 4 h[29].

- Systemic distribution of insulin has been demonstrated following absorption from the vagina in rats and rabbits, so this may be a potential alternative route for delivery of currently available, 'injection-only' biopharmaceuticals[30]. Insulin is a hydrophilic molecule that is absorbed through intercellular aqueous channels; hence, absorption would be greater when the epithelium is thinner or by using a penetration enhancer. It has been found that there is an increase in hypoglycaemia in rats when insulin was administered vaginally using effective enhancer such as sodium taurodihydrofusidate and polyoxyethylene-9-lauryl ether[31].

DNA-based vaccines are being investigated to overcome the deficiencies of antigen-based vaccines for the treatment of HIV and vaginal mucosa was considered as one of the effective routes of administration[32].

4.3 Vaginal local drug delivery

Drugs are often required to be effective locally and with minimal systemic absorption.

- In a study in patients with vaginal rhabdomyosarcoma and residual vaginal disease following surgery and chemotherapy, the effect of high-dose irradiation from vaginal moulds loaded with iridium were examined and it was found that patients treated with these vaginal preparations remained well and disease free for 7 years[33].

- A number of studies have investigated the local delivery of antimicrobial agents. Chitosan/alginate complexes have been investigated for the preparation of vaginal inserts containing chlorhexidine digluconate for local treatment of genital infections. On the basis of insert water uptake and chlorhexidine digluconate release, inserts based on the complex CH/ALG(1:9) were found to provide the optimum drug release with the efficiency to kill the principal pathogens responsible for aerobic vaginitis and candidiasis[34]. Liposomal local vaginal delivery of acyclovir, metronidazole and clotrimazole in the form of bioadhesive gels can provide an excellent means for treatment of vaginal infections[35,36]. In an investigation on the local delivery of itraconazole, bioadhesive film made up of hydroxypropylmethyl cellulose and hydroxypropyl cellulose based system it has been reported that these films were able to deliver the drug successfully for 8 h. During lactobacillus and cytotoxicity studies it has been concluded that bioadhesive films can be used intravaginally without affecting cell viability of the vaginal mucosa[37]. In a clinical study on 136 patients, it has been found that single application of butoconazole 2% cream for the treatment of vaginal

candidiasis was more effective than the fluconazole 150 mg oral tablets to get the relief from the signs and symptoms of vaginal candidiasis compared[38].

- Local delivery of lyophilized *Lactobacillus sporogenes* in the form of a vaginal suppository has been recently studied as a candidate for probiotic prophylaxis and treatment of vaginal infections by restoring the natural vaginal flora to a healthy state. The cocoa butter suppositories used in the experiment dissolved at the application site at the body temperature and the without affecting the viability of *Lactobacillus sporogenes*[39].

Table 2: Intravaginal formulations available in the market for the treatment of STIs and vaginal infections

Vaginal formulations	Brand name	Active ingredients	Intended use
Gels and creams	Femstat Gynazol 1	Butaconazole	Antifungal
	Mycelex 3 Gyne-Lotrimin Gynex Trivagizole	Clotrimazole	
	Monistat Leader miconazole 1	Miconazole	
	Terazol	Teraconazole	
	Vagistat	Tioconazole	
	Sporanox	Itraconazole	
	Diflucan	Fluconazole	
	Nilstat	Nystatin	
	Econate VT	Econazole	
	Flagystatin	Metronidazole+ Nystatin	Mixed infection (Trichomonas, Candida) Bacterial vaginosis
	Metrogel Metrocream Vandazole	Metronidazole	Bacterial vaginosis
	Vivagel	SPL7013, or astodrimer sodium	
Vaginal suppositories/ pessaries	Terazole 3	Terconazole	Antifungal
	Gyno-Pevaryl	Econazole nitrate Clotrimazole	
	Canesten	Adipic acid	
	Myclo-gyne	Clindamycin	
	Cleocin		Bacterial vaginosis
Vaginal ovule	Gynecure	Tioconazole	Antifungal
	Flagystatin	Metronidaole+ Nystatin	Trichomonas & Candida infection

5. Vaginal formulations

Vaginal drug delivery formulations span the spectrum from semi-solids to solid dosage forms and are capable of short-term drug delivery over several hours (Table 2) to sustained release over several months (intravaginal rings, Table 3). Some of these conventional preparations are criticised due to their inconvenience of multiple days of drug dosing (vaginal tablets), messiness and leakages (vaginal creams and gels), the requirement of applicators and sometimes if any product with reduced or single-day treatment is used then it may contain increased drug concentration.

6. Intravaginal rings

Intravaginal rings (IVRs) are flexible, elastomeric devices of approximately 5.5 cm in diameter and a cross-sectional diameter of 4-9 mm. These rings offer long-term controlled, sustained drug delivery, which is advantageous over other conventional vaginal drug delivery. The controlled delivery of microbicides from IVRs is also advantageous over semi-solid vaginal formulations, for their longer duration, and reduced frequency self-administration, which increases the adherence of patients to their treatment regimen. IVRs are widely used for contraception (Table 3), with high levels of efficacy and user acceptability[40]. There are very few chances of clinically significant lesions with IVRs and women have a greater preference for IVRs with applicators and a one size fit-free diaphragm for drug delivery[41]. Other than the commonly available IVRs (Table 3) a few other IVRs for the prevention of HIV are under development (Table 4).

Table 3: Intravaginal rings that are commercially available

Name of ring	Active ingredients	Polymer used	Uses
Nuvaring®	Etonogestrel + ethinyl estradiol	Polyethylene-co-vinyl acetate	Contraception
Femring®	Estradiol-3-acetate	Silicone	Relief of vaginal and urogenital symptoms in menopausal women
Estring®	Estradiol	Silicone	Relief of vaginal and urogenital symptoms in menopausal women
Progering®	Progesterone	Silicone	Contraception in lactating women
Fertiring®	Progesterone	Silicone	Contraception

In general, IVRs avoid the multiple days of dosing and avoid messiness and leakage associated with conventional systems such as creams and gels[42]. Some disadvantages do exist, however, such as the potential for the expulsion of rings**,** discomfort due to the feeling of an exogenous material inside the vagina and occasional side effects such as bleeding and itching. So before designing a new IVR careful consideration of dimension to reduce the chances of expulsion and suitable choice of polymer is very important to minimise the discomfort cause by these IVRs.

6.1 Types of intravaginal rings

6.1.1 Matrix rings

This is the most common and simple design of an IVR, in which drug particles are dispersed uniformly throughout the polymer (Figure 4A). The drug particles present on the surface dissolve when it comes to contact with vaginal fluid. With time, vaginal fluid enters the pores and channels formed when drug molecules dissolve and further release takes place. As drug release takes place, the thickness of the zone of drug depletion increases and the diffusion path length for the rest of the drug increases, which leads to decrease in drug release with time. The drug release behaviour for this type of ring system can be best explained by the Higuchi equation[43-45],

$$Q = (Dp\,[2A - Cp]Cp\,t)^{0.5}$$

where Q is cumulative release per unit surface area (mg/cm^2), D_p is the drug diffusion coefficient in the polymer (cm^2/day), A is the drug loading per unit volume in the polymer (mg/cm^3), C_p is the drug solubility per unit volume in the polymer and t is time (days)[43].

6.1.2 Reservoir vaginal rings

In this type of design, a non-medicated polymeric sheath surrounds a core that contains the drug (Figure 4B). In this type of system, the thickness of the non-medicated sheath controls the rate of drug release. The medicated core contains uniformly distributed drug which diffuses out through the non-medicated sheath with constant rate “zero order” release kinetics. A modification of this design is the shell-type IVR in which the drug core is sandwiched between a non-medicated core and outer non-medicated sheath[44].

Other than the above designs, a few other novel IVR designs have been suggested particularly to overcome the problem of delivery of hydrophilic, high molecular weight and

temperature labile drugs through hydrophobic polymers. In the modified designs, the ring body acts as the holder and solid dosage forms are inserted in the holder for delivery.

6.1.3 Coated pod insert IVR

A non-medicated polymeric ring contains coated drug pods incorporated in polymeric elastomer (Figure 4C). The diameters of the delivery window have a major effect on the drug delivery. Other factors that affect drug delivery are the polymer used for IVR production and the number of inserted pods[46].

6.1.4 Rod and tablet insert IVR

This system is same as that of coated pod inserts but in this system instead of coated pods it contains lyophilised polymeric gel or compressed tablets[47].

6.1.5 Multi segment IVR

This design is useful for the delivery of two or more drugs simultaneously without the need to mix the drugs. Drugs can be loaded individually to avoid any cross reactivity[48].

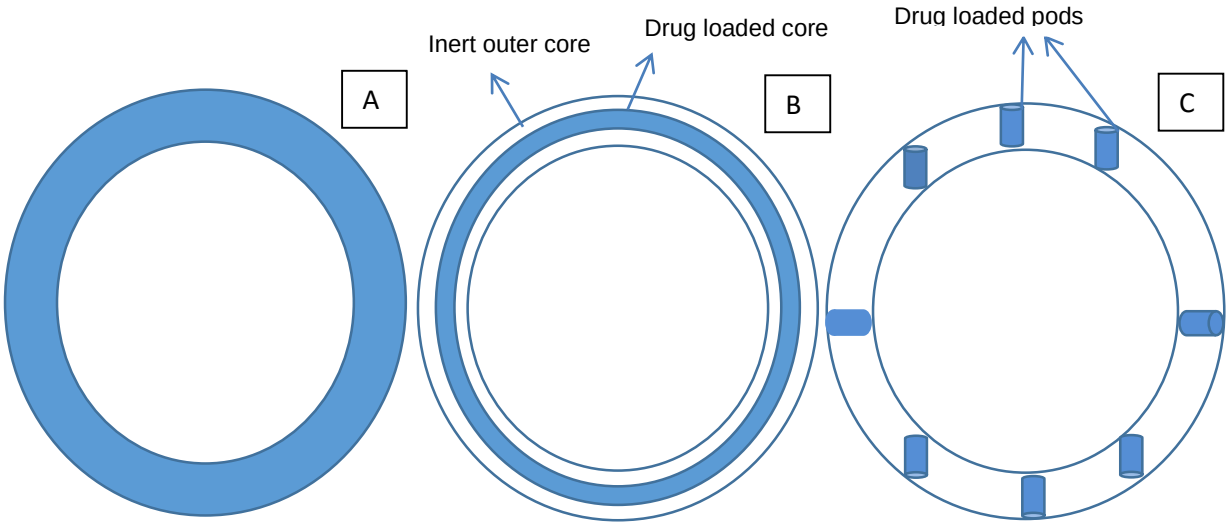

Figure 4: Intravaginal ring designs: A) Matrix B) Reservoir C) Pod type. Blue colour represents the part of IVR loaded with drug and white colour represents without drug

IVRs have been investigated for delivery of various drugs for HIV prevention (Table 4), contraception or to relieve menopausal symptoms.

Table 4: Intravaginal rings investigated for the prevention of HIV

Drugs	Polymeric material	Ring design	References
Dapivirine	Silicone	Reservoir,	[49]
	Silicone	Matrix	[50]
	Polyurethane	Matrix	[51]
	Acacia gum	Reservoir	[52]
CMPD 167 + maraviroc	Silicone	Matrix	[53]
MC 1220	Silicone	Matrix	[54]
TMC 120 + Boc-LBA	Acacia gum	Reservoir	[52]
TMC 120 + PMPA			
Tenofovir	Polylactide and polyethylene vinyl acetate blends	Matrix (Rods)	[55]
	Polyurethane	Reservoir	[56]
MIV-160	Poly(ethylene-co-vinyl acetate	Matrix	[57]
UC781	Silicone	Matrix	[58]
	Polyurethane	Matrix	[59]
	Poly(ethylene-co-vinyl acetate)	Matrix	[58]
Dapivirine + tenofovir	Acacia gum	Reservoir	[52]
	Polyurethane	Segmented	[59]
Dapivirine + maraviroc	Silicone	Matrix	[43]
Tenofovir + acyclovir	Silicone elastomer	Pod ring	[60]
IQP	Polyurethane	Matrix	[61]
Dapivirine + darunavir	Silicone	Matrix	[62]
Levonorgestral + tenofovir	Polyurethane	Segmented	[48]

6.2 Acceptability studies of intravaginal rings

Before using IVRs for any treatment purpose it is very important to know whether the IVRs are acceptable by women as a treatment strategy. Many studies have been conducted to check the acceptability of these rings, mostly with contraceptive devices. Efficacy, acceptability and tolerability of contraceptive rings containing etonogestrel and ethinyl oestradiol were tested with 1145 women. During the study period, each woman used the ring for 3 weeks and it was reported that out of all the women who completed the study 96% were satisfied with the ring, 98% of them recommended this to others and 91% complied with the prescribed regimen[63] In a tolerability and user acceptability study of novel combined contraceptive IVRs for up to 13 cycles with 2015 women, 85% of women who completed the study were satisfied with the ring and 90% recommended it as an acceptable method to others and only 2.5% discontinuation was reported due to discomfort caused by the device[40] so rejection of IVRs on the basis of discomfort is low. A multicentre study conducted on 1950 women involved a 21-item questionnaire regarding ease of use of vaginal rings, clarity of instructions, sexual comfort, satisfaction, cycle-related characteristics and compliance etc. More than 95% of women who completed the study were satisfied or very satisfied and would recommend the ring to others, and the 60% of the women who discontinued the study were satisfied or very satisfied and would recommend the ring to others[64].

A multicentre randomized controlled trial has been conducted on 500 women to compare the IVR to vaginal patch for contraception. Three cycles of use resulted in 228 of 241 (94.6%) and 210 of 238 (88.2%) women for ring and patch respectively completing the study. 71% of women who used the ring planned to continue that method after the study, whereas only 26.5% wanted to keep using the patch[65]. In a comparison study with 80 women, IVRs were compared with oral dosage form for the purpose of contraception. The average score for ring acceptability was 4.3 ± 0.9 out of 5 for the ring in comparison with the 3.6 ± 1.0 for oral contraceptives; not only were these rings more acceptable, they also increased the concentration of H_2O_2 producing lactobacilli species by 2.7 fold[66]. This means that these IVRs are not only highly acceptable in comparison to other commonly used vaginal products but they also strengthen the vaginal defence by increasing the number of vaginal lactobacilli. A review of articles on contraceptive ring use indicated that the rings are highly acceptable and 90% of the women under study didn't have any problem with the ring and didn't feel the ring inside the vagina[67].

Apart from contraception, IVRs have also been studied for hormonal drug delivery for other purposes. An acceptability study of estradiol IVRs over vaginal creams reported that 47% of the 83 patients randomized to be treated with the IVRs gave the opinion 'excellent' as a method to relieve vaginal atrophy and dryness as compared with 6% of the 82 patients using a vaginal cream. 78% of patients treated with the ring answered 'good' or 'excellent' as compared with 40% with the cream[68]. In terms of studies specific to antimicrobial agent delivery, a smaller number of studies have been conducted. An adherence study with 405 couples compared three different types of vaginal preparations for the delivery of a microbicide (diaphragm, IVRs and applicator). 52.9% of the women preferred vaginal rings, 36.4% preferred applicators and 10.7% selected the diaphragm[69] so IVRs were the preferred option for drug delivery over other commonly used vaginal products for the delivery of microbicides. A study was performed with 157 young African women involving the use of placebo silicone vaginal rings for 12 weeks with the expectation for use for the purpose of HIV prevention. During 12 weeks, women found the IVR highly acceptable due to its continuous use which could allow spontaneous protection against HIV and their non-interference with sexual intercourse. 67% of female participants were concerned about interference during intercourse that could be unacceptable to their partners, so using the right dimensions for a ring can increase confidence in IVR drug delivery by decreasing the probability of interference during intercourse[70]. This last study is important because acceptability of these rings in developing countries must be considered; as due to reduced access to healthcare facilities the chances of STI infection is greater compared to developed countries[70], so IVRs can be a breakthrough in reducing the cost of treatment and decreasing the chances of reinfection.

7. Polycaprolactone

7.1 Polycaprolactone as a potential material for IVRs

Polycaprolactone (PCL) was one of the earliest polymers synthesized by the Carothers group in the early 1930s and became commercially available. It is semi-crystalline, synthetic, aliphatic polyester synthesised by ring opening polymerisation of the ε-caprolactone monomer.

PCL is an attractive polymer for IVR production due to its flexibility and high extensibility[71]. It has an extremely low glass to rubber transition temperature (Tg) of around -60°C, which makes the material highly suitable for melt processing[72]. At room temperature, PCL is highly soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene,

cyclohexanone and 2-nitropropane; slightly soluble in acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile; and insoluble in alcohols, petroleum ether, diethyl ether and water[73]. PCL has been extensively used for the production microcapsules and matrix-type drug delivery devices by solution processing techniques due to its solubility properties.

PCL can be enzymatically biodegraded by microorganisms into non-toxic products but it takes several months or years to degrade it completely. Biodegradation takes place by hydrolysis of ester linkages under physiological conditions. Hydrolytic degradation of PCL involves surface and bulk degradation. In surface degradation, hydrolytic cleavage of the polymer chain leads to the reduction in viscosity without affecting the molecular weight. In the second stage, bulk degradation of PCL occurs, characterised by a decrease in molecular weight. Once the molecular weight has decreased to 3000 Da or less PCL may be completely resorbed and degraded by intracellular mechanisms[74].

7.2 The application PCL in drug delivery system

Over the last few decades, PCL polymers have been of major interest for developing a variety of controlled drug delivery systems in the form of nanospheres, microspheres, fibres and matrices[75,76]. PCL matrices have been produced for the delivery of hydrophilic drugs such as gentamicin sulphate[77] and vancomicin[78], hydrophobic compounds including progesterone[79], and for the delivery of macromolecules (catalase, lysozyme and collagenase)[80]. The precipitation casting technique for PCL matrix production has been established for controlled release of small molecule antibacterial (gentamicin sulphate)[77], steroids (progesterone)[79] and macromolecules (inulin)[81]. A layer of methanol is added to PCL solution containing dissolved or dispersed drug and gradual precipitation of the PCL phase results by solvent transport across a polymer film formed at the solution/non-solvent interface. The final step involves drying the resulting matrices under ambient conditions[77]. The rapid cooling technique has recently been developed for controlled delivery of proteins, enzymes[80] and microbicides[82] from PCL matrices. This technique offers advantages over precipitation casting or film casting/solvent evaporation approaches since drug particles can be dispersed effectively throughout the matrix by rapid cooling-induced crystallisation of the PCL phase. Particle sedimentation leading to poor drug distribution in the matrix is thereby avoided. Table 5 represents the various drugs loaded into PCL matrices and the methods used for their preparation.

Table 5: Drug delivery from PCL matrices

Loaded Drugs	Preparation method	Time of release (days)	References
Lysozyme	Rapid cooling	12	[80]
Collagenase	Rapid cooling	18	[80]
Catalase	Rapid cooling	15	[80]
Lactose	Precipitation cast	3	[83]
Gentamicin sulphate	Matrices	14	[77]
Gelatin	Matrices	21	[83]
Progesterone	Co-dissolution	10	[79]
Vancomycin	Microencapsulation	2	[78]
Miconazole	Rapid cooling	13	[82]
Ciprofloxacin	Rapid cooling	30	[82]
Inulin	Precipitation		[81]

PCL could be a potential polymer for the production of IVRs because of its low processing temperature, biocompatible and biodegradable nature, and highly porous morphology. Nhung *et al.* previously demonstrated the use of PCL polymer for the delivery of antiviral agents mainly focused toward intravaginal prevention of HIV. She also considered the delivery of the antibacterials, ciprofloxacin and miconazole using PCL for intravaginal use[84]. The mechanical characteristics of PCL are usually reported in the literature for solid PCL or for the PCL scaffold[22,85]. However, *Wang et al.* reported the potential of microporous PCL matrices for the production of tubes for soft tissue engineering[84]. In the literature, it is evident that PCL can be used for the delivery of many drugs by using various processing techniques (Table 5) so the focus here is PCL as a potential material for the delivery of antimicrobial agents intended for the intravaginal treatment of STIs.

8. Conclusion

It is clear that IVRs are highly acceptable to women in terms of convenience, avoidance of messiness associated with creams and gels, reduced frequency of dosing and the personal control offered by the vaginal ring. Additionally, drug levels in the blood can be greater than results from orally administered preparations[66] indicating that this is an option for systemic drug delivery. The use of these systems is still limited to contraception and HIV prevention so further exploration is required into their potential use in the treatment of other conditions. STIs, which are an exceptionally important problem for women's health, could be an area in which IVRs can be used as a treatment strategy. The topical and

sustained release of drugs from vaginal rings can provide direct and immediate relief and reduce the chances of reinfection. High levels of patient acceptability increases the chances of completion of therapy, which would reduce the chances of developing drug resistance in the microorganisms. Due to disadvantages of current polymers as described above PCL offer many advantages as a potential polymer for the delivery of antibacterials for the treatment of STIs. Low processing temperature, biodegradability and biocompatibility offered by PCL make it a favourable polymer for the investigation of IVR.

9. Hypothesis

Based on the preceding literature review, the overarching hypothesis for this thesis is that polycaprolactone is a suitable polymer for intravaginal delivery of antibacterials against the organisms that cause sexually transmitted infections.

10. Aims

Metronidazole is one of the most prescribed medications for the treatment of bacterial vaginosis, and vaginal preparations of metronidazole for the treatment of bacterial vaginosis are already available in the market. However the creams, gels and pessaries require daily dosing and can leak from the vagina, so improved vaginal delivery would be very useful. Therefore, the first aim of this thesis is to investigate the formulation of PCL matrices containing metronidazole for the treatment of bacterial vaginosis. Chapter 2 addresses this first aim, by using a published method for the preparation and loading of PCL matrices based on rapid cooling suspensions of drug powder in PCL solutions in acetone and subsequently adjusting polymer properties and drug loading by incorporating PEG.

Doxycycline is a commonly prescribed medication for the treatment of STIs such as chlamydia, gonorrhoea, lymphogranuloma and syphilis. There are currently no studies focused on the delivery of doxycycline intravaginally, so the second aim of this thesis is to investigate the loading and delivery of doxycycline from PCL for use as an IVR (Chapter 3). Additionally, in this chapter the biocompatibility of PCL for vaginal delivery is by assessed in terms of *in vitro* toxicity against a vaginal cell line and the normal commensal bacteria, *Lactobacillus jensenii,* in cell culture.

The combination of metronidazole and doxycycline is the most widely accepted treatment for pelvic inflammatory disease. Combining of drugs with different modes of action within a single vaginal ring has been tried previously for HIV prevention but not for the treatment of

STIs. Therefore Chapter 4 addresses the third aim of the thesis, which is to load the combination of metronidazole and doxycycline in the PCL matrices for the treatment of pelvic inflammatory disease and to check the effect of the combination of drugs on polymeric properties, and on drug loading and release.

Chapter 5 deals with the fourth aim of the thesis, which is to test the PCL polymer for the potential to deliver a macromolecule, because recently many macromolecular drugs of biotechnological origin have been studied for the treatment and prevention of STIs.

In order to address the aims described above, within each of the experimental chapters a series of experiments are performed:

A) To optimise the formulation conditions for PCL matrices.

B) To characterise the matrices in terms of drug loading and physicochemical properties include hardness and morphology.

C) To observe the effect of drug loading on the morphology of the PCL matrix using scanning electron microscopy and differential scanning calorimetry.

D) To investigate the *in vitro* release behaviour of antimicrobials from PCL matrices.

E) To determine the level of antibacterial activity of drug released from the PCL matrices.

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CHAPTER 2

EVALUATION OF POLYCAPROLACTONE MATRICES FOR THE DELIVERY OF METRONIDAZOLE IN THE INTRAVAGINAL TREATMENT OF BACTERIAL VAGINOSIS

1. Abstract

Microporous, poly (ε-caprolactone) (PCL) matrices loaded with the antibacterial metronidazole were produced by rapidly cooling suspensions of drug powder in PCL solutions in acetone. Drug incorporation efficiency of 40-53% was obtained on raising the drug loading from 5-20% w/w measured with respect to the PCL content. Rapid 'burst release' of 35 to 55% of the metronidazole content was recorded over 24h when matrices were immersed in simulated vaginal fluid (SVF), due to the presence of large amounts of drug on matrix surface as revealed by RAMAN microscopy. Gradual release of around 80% of the drug content occurred over the following 12 days. Metronidazole released from PCL matrices in SVF retained antimicrobial activity against *Gardnerella vaginalis in vitro* at levels up to 97% compared to the free drug. On the basis of these results, the 15% w/w metronidazole loading was selected to check the effect of polyethylene glycol (PEG) on drug loading, release and mechanical properties of PCL. Incorporation efficiency of metronidazole increased from 54% to 91% as the concentration of PEG increased from 0% to 10% w/w with respect to PCL. Release of metronidazole within 24 hours of immersion in simulated vaginal fluid at 37°C increased from 48 to 76% as PEG loading increased from 0% to 10% w/w, and overall release within the 12 day experimental period was greater for 10% PEG (95% drug release) in comparison to 0% PEG (76% released). Released metronidazole retained a high relative antibacterial activity of 88-97% and there was no discernible effect of PEG on the antimicrobial activity of metronidazole according to a disc diffusion assay against *G. vaginalis*. Basic modelling predicted that the concentrations of metronidazole released into vaginal fluid *in vivo* from the approximate quantity of PCL matrix that would be used in an intravaginal ring (IVR) would exceed the minimum inhibitory concentration (MIC) of metronidazole against *G. vaginalis*.

2. Introduction

Bacterial vaginosis (BV) is one of the most common genital conditions occurring in women of child bearing age[1] and is caused by the displacement of normal vaginal lactobacilli by other species notably *Gardnerella vaginalis*, *Mycoplasma homonis* and anaerobic bacteria such as peptostretococci, *Prevotella* spp. and *Mobiluncus* spp[2]. BV is associated with a range of health problems such as altered vaginal discharge and odour. It is also related with the adverse pregnancy outcomes including post-partum, post-abortion, and post-hysterectomy infections. BV increases women's risk of acquiring pelvic inflammatory disease and potentially some sexually transmitted infections (STIs) such as *Chlamydia*

trachomatis, *Neisseria gonorrheae* and HIV by inducing changes in the mucosal immune environment of the vagina[2,3,4]. BV is estimated to have a mean prevalence of 14% when considering both developed and developing countries, but as the microflora of the vaginal ecosystem changes throughout the menstrual cycle, under the influence of exogenous hormones and during reproductive life in most women it is difficult to estimate the true prevalence or impact of BV[5]. In the United States, bacterial vaginosis affects approximately 80,000 pregnant women per year, resulting in an increased incidence of preterm delivery or low-birth weight. Treatment can reduce these risks and may in turn reduce the number of associated perinatal deaths and neurologic abnormalities in infants[6].

BV, when treated, is generally managed by using metronidazole, which belongs to the nitro-imidazole class of antibiotics and exhibits broad spectrum activity against most Gram-negative and Gram-positive anaerobic bacteria[7]. Metronidazole is particularly attractive for the treatment of BV because it also eradicates any coexisting trichomoniasis, an STI caused by the protozoa, *Trichomonas vaginalis*[3]. Metronidazole is generally administered orally in tablet form (2 g single dose or 400 mg, 12-hourly for 7 days) but this route is often associated with adverse gastrointestinal side effects, nausea, headache, anorexia and occasionally vomiting. The bitter or metallic taste of oral dosage forms presents a further disadvantage.

The side effects of metronidazole have been avoided by vaginal administration. Unfortunately, solid vaginal formulations such as pessaries and tablets have a short residence time, necessitating frequent administration[8]. Semi-solid intravaginal gels incorporating metronidazole (0.75%) require daily administration of approximately 5 g gel for 5 days but are messy to apply, prone to leakage and concerns exist over effective coverage of the vaginal epithelium[9]. These factors have contributed to the recent upsurge of interest in intravaginal ring (IVR) devices for sustained delivery of antiviral agents[10]. IVRs offer advantages of low and continuous dosing over extended time periods, reduced side effects, self-administration and improved patient compliance. Conventional IVRs produced from silicone elastomer or poly (ethylene vinyl acetate) (pEVA) have been used clinically for many years for delivery of estrogen (hormone replacement therapy) and etonogestrel and ethinyl estradiol (contraceptive purposes)[11]. IVRs are being evaluated for sustained release of the non-nucleoside reverse transcriptase inhibitor, dapivirine as an anti-HIV microbicide and acyclovir for herpes prophylaxis[12,13].

Conventional IVRs do however display a number of disadvantages for microbicide delivery; they are generally restricted to delivery of low molecular weight, hydrophobic drugs such as dapivirine. In addition, manufacture involves heating at 80°C for silicone elastomer or 140°C for pEVA which could degrade thermally-sensitive compounds. The synthetic polyester, poly (ε-caprolactone) (PCL) does not require such high temperatures as part of drug loading or shape moulding. PCL has been investigated extensively for many years for production of a range of drug delivery systems including microparticles, nanoparticles, films and fibres[14]. PCL nanoparticles loaded with the immunosuppressant cyclosporin, for example, have been reported to decrease the nephrotoxicity of the drug and efficiently target lymphocytes[15]. Microporous PCL matrices, prepared by precipitation casting[16] or rapid cooling techniques[17] are effective for sustained delivery of small hydrophobic drug molecules (progesterone)[18], hydrophilic entities (gentamicin sulphate)[16] and macromolecules such as enzymes with retained activity[19]. Recently, the potential utility of PCL in vaginal delivery was demonstrated for the antibacterial, ciprofloxacin, with loadings of 7.3-15% w/w, and the antifungal, miconazole (loadings of 1-3% w/w). Drug released into simulated vaginal fluid retained high antibacterial activity against *N. gonorrhoeae* and *C. albicans*, respectively[20].

In the present study the loading and release of metronidazole from PCL matrices is investigated. The potential to adjust metronidazole loading, release and mechanical properties of PCL with the addition of polyethylene glycol (PEG) is also considered. PEG is amongst the most commonly used water soluble pore forming agents (or porogens) that is incorporated into drug delivery systems to enhance the rate of drug release[21-23] and is safe for vaginal delivery[28]. The presence of PEG increases pore formation by solubilisation and extraction of the PEG phase and thus is expected to facilitate fluid uptake, improving the loading, dissolution and release of co-incorporated drugs[24-27].

3. Materials and methods

3.1 Materials

PCL (Mw 115,000 Da, CAPA 6500) was obtained from Solvay Interox, Warrington, UK. Metronidazole, polyethylene glycol (MW 1500), glucose, urea, bovine serum albumin, potassium hydroxide, calcium chloride, glycerol, lactic acid and acetic acid were purchased from Sigma-Aldrich, Australia. *G. vaginalis* stock culture in glycerol broth was supplied by Micromon, Monash University, Clayton, VIC, Australia. Horse blood agar,

heart infusion broth (HIB), CO_2 generation kit and antimicrobial susceptibility blank discs were obtained from Oxoid, Basingstoke, UK.

3.2 Production of metronidazole-loaded PCL matrices

PCL solutions of concentration 15% w/v were prepared by dissolving the polymer in acetone at 50°C. Metronidazole was ground to a fine powder and added to the PCL solution to produce suspensions of concentrations 5, 10, 15 and 20% w/w of the PCL content. The resulting suspension was homogenized for 30s at 5000 rpm using a Silverson SL27 homogenizer (Silverson Machines, Chesham, Bucks, UK). The suspension was poured into a polypropylene syringe body (3 mL) which was used as a mould and cooled in ethanol at -80°C for 2 h to allow crystallization of PCL. The hardened matrices were removed from the moulds and immersed in 10 mL ethanol for 24 h to extract acetone by solvent exchange. Samples were removed from ethanol and left to dry under ambient conditions to evaporate residual solvents. The final matrices were in the form of cylinders of diameter 6.5 ± 0.5 mm and length 45.0 ± 5 mm.

PEG was additionally incorporated into the PCL matrices in the same way. 15% w/v PCL matrices loaded with combination of metronidazole (15% w/w) and PEG (0, 2, 3, 4, 5 and 10% w/w) were prepared. The final PCL/PEG matrices were in the form of cylinders of diameter 6.5 ± 0.2 mm, length 45.0 ± 7 mm and weigh (410 ± 15 for PCL) (415 ± 16 for PCL/PEG) matrices.

3.3 Determination of metronidazole content

Samples of PCL and PCL/PEG matrices loaded with metronidazole were weighed and dissolved in 2 mL of dichloromethane (DCM). Precipitation of PCL was induced by adding 5 mL of 30% methanol followed by shaking at 1000 rpm overnight (Vibrax VXR, IKA, Werke Staufen, Germany) to evaporate DCM and obtain partitioning of the drug into the methanol phase. The concentration of metronidazole in methanol was measured by using UV spectrophotometry (Varian, Cary 50 Bio, Agilent Technology, USA) at an absorbance wavelength of 319 nm and calculated using a calibration curve of metronidazole in 30% methanol (4-20 µg/mL). Experiments were performed in triplicate to obtain values of actual drug loading and loading efficiency.

3.4 Morphology

The morphology of the surface and interior of drug-free and drug-loaded PCL and PCL/PEG matrices was examined using a JSM 6460LA scanning electron microscope (SEM, JEOL, Japan). Specimens were mounted on aluminium SEM stubs using carbon tabs and sputter coated with platinum using an Eiko-Sputter coater automatic mounting press, prior to examination at a voltage of 5 kV.

3.5 Thermal properties

The thermal characteristics of matrices were investigated using differential scanning calorimetry (DSC) (DSC 1 STARe System, Mettler Toledo, Switzerland) under a nitrogen atmosphere. Samples of matrices were weighed, placed in sealed aluminium pans and heated over the temperature range -100°C to 150°C at a rate of 10°C/min. The peak melting point, glass transition temperature (Tg) and heat of fusion data were obtained using the DSC software facility. Crystallinity (%) was estimated using a value of 139.5 J/g for the heat of fusion of fully crystalline PCL[18].

3.6 Shore hardness testing

Shore hardness testing was carried out using a CT3 Texture Analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA). As-moulded cylinders were mounted horizontally and compressed locally at a speed of 0.1 mm/min to a depth of 2.0 mm using a 2 mm diameter, flat-ended, cylindrical probe (TA39). The hardness (or indentation resistance) of each sample was calculated from the applied force measured at a depth of 2 mm. A pEVA IVR (Nuvaring®, Schering-Plough Pty limited, NSW, Australia) was subjected to the same test procedure for comparison.

3.7 *In vitro* release of metronidazole

Cylindrical sections of metronidazole-loaded matrices (length 45 mm) were subjected to a release study in simulated vaginal fluid. Prior to testing, both ends of each sample were sealed by dipping in 5% w/v solution of PCL in acetone followed by drying in air. Experiments were performed in triplicate. Each sample was placed separately in 10 mL of SVF and retained at 37°C in an incubator. SVF was prepared according to the method of Owen and Katz[29] and contained 3.51 g NaCl, 1.40 g KOH, 0.222 g $Ca(OH)_2$, 0.018 g bovine serum albumin, 2.00 g lactic acid, 1.00 g acetic acid, 0.16 g glycerol, 0.4g urea and 5.0 g glucose up to 2 L of distilled water. The pH was adjusted to 4.2 using 10% HCl. The

release media were collected and replaced with fresh media daily for 12 days. The concentration of drug in the release medium was analysed by UV spectrophotometry (Varian, Cary 50 Bio, Agilent technology, USA) at 319 nm by comparison with a standard curve produced using a series dilution of metronidazole in SVF. Separate release samples were stored at 4°C for antimicrobial testing.

3.8 Analysis of drug distribution

Raman spectroscopy was used to map 10% w/w metronidazole-loaded PCL matrices to characterise the drug distribution for correlation with drug release behaviour. A 1 mm thick disk was taken from the middle of drug-loaded PCL matrices, placed on a glass slide and scanned using a Raman microscope (Nicolet Almega XR Dispersive Raman, Thermoscientific, USA) along the radius or around the circumference at spatial intervals of 100 µm.

3.9 *In vitro* assay of antimicrobial activity

The antimicrobial activity of metronidazole released from the matrices was assayed with *G. vaginalis* using the disc diffusion method. *G. vaginalis* was stored as stock cultures in 40% glycerol at -80°C. *G. vaginalis* was grown for 48 h at 37°C on horse blood agar plates and the colonies of bacteria were then scraped from the agar surface using a speader and heart infusion (HI) broth. The cell suspension was then diluted in HI broth and plated onto horse blood agar plate to get approximately 100 CFU per plate. A blank disc (Oxoid) was placed at the centre of each inoculated plate and 100 µL of drug standard solution in SVF or release medium containing metronidazole was added to the disc. The plates were incubated at 37°C for 48 h under anaerobic conditions and the diameter of the zone of inhibition surrounding the disc was measured. The relative antibacterial activity of metronidazole released from PCL matrices was calculated by comparison with the zone of inhibition obtained using non-formulated drug solutions of metronidazole of the same concentration. SVF and release media used for incubation of blank PCL and PEG/PCL matrices were used as controls.

3.10 Statistical analysis

Significant differences between samples in their drug loading, drug release on day 1 and day 14, glass transition temperature, crystallinity and shore hardness were tested using one-way ANOVA with a Tukey multiple comparisons test ($p<0.05$).

4. Results and Discussion

4.1 Morphology of matrices

Metronidazole-loaded PCL matrices prepared by rapidly cooling suspensions of drug powder in PCL solution exhibit flexibility, uniformity of structure and an absence of large cracks and voids in the sample surface and interior. SEM examination of drug-free PCL matrices revealed a nodular type of morphology and irregular shaped pores with dimensions of 2-4 μm (Figure 1a). The surface of metronidazole-loaded matrices exhibited a flat texture (Figure 1b), probably resulting from contact of the matrix with the mould wall. Fine fissures were observed in certain areas along with evidence of trapezoidal-shaped drug crystals 1-2 μm in size. The internal structure of metronidazole-loaded PCL matrices exhibited a woven, lamellar type of morphology (Figure 1c) and the characteristic microporous PCL phase consisting of 2-5 μm pores. Trapezoidal drug crystals are visible at higher magnification (Figure 1d, arrowed). The addition of PEG into matrices was associated with larger pore sizes (Figure 1e).

4.2 Metronidazole loading

Actual loading of metronidazole in PCL matrices was lower than the corresponding theoretical loading (Table 1), with approximately 50% of the quantity added to the mixture being incorporated. This is explained by the relative solubility of metronidazole in the solvent used to extract acetone from the hardened PCL matrices, required to avoid shrinkage and cracking of the PCL matrix following crystallization and drying, which results in partitioning of metronidazole and elution from the matrix. The solubility of metronidazole in methanol, acetone and ethanol is 32.2, 20.7 and 5.0 mg/ml[30], respectively. Methanol was also found to be an issue for preparation of ciprofloxacin- and miconazole-loaded PCL matrices, leading to 73% and 20% loading efficiency, respectively[20]. Ethanol instead of methanol for acetone extraction resulted in an increase in catalase loading in PCL matrices[19] due to the lower solubility of the enzyme in ethanol, so ethanol was used for this stage instead of methanol in the present study in order to reduce drug loss.

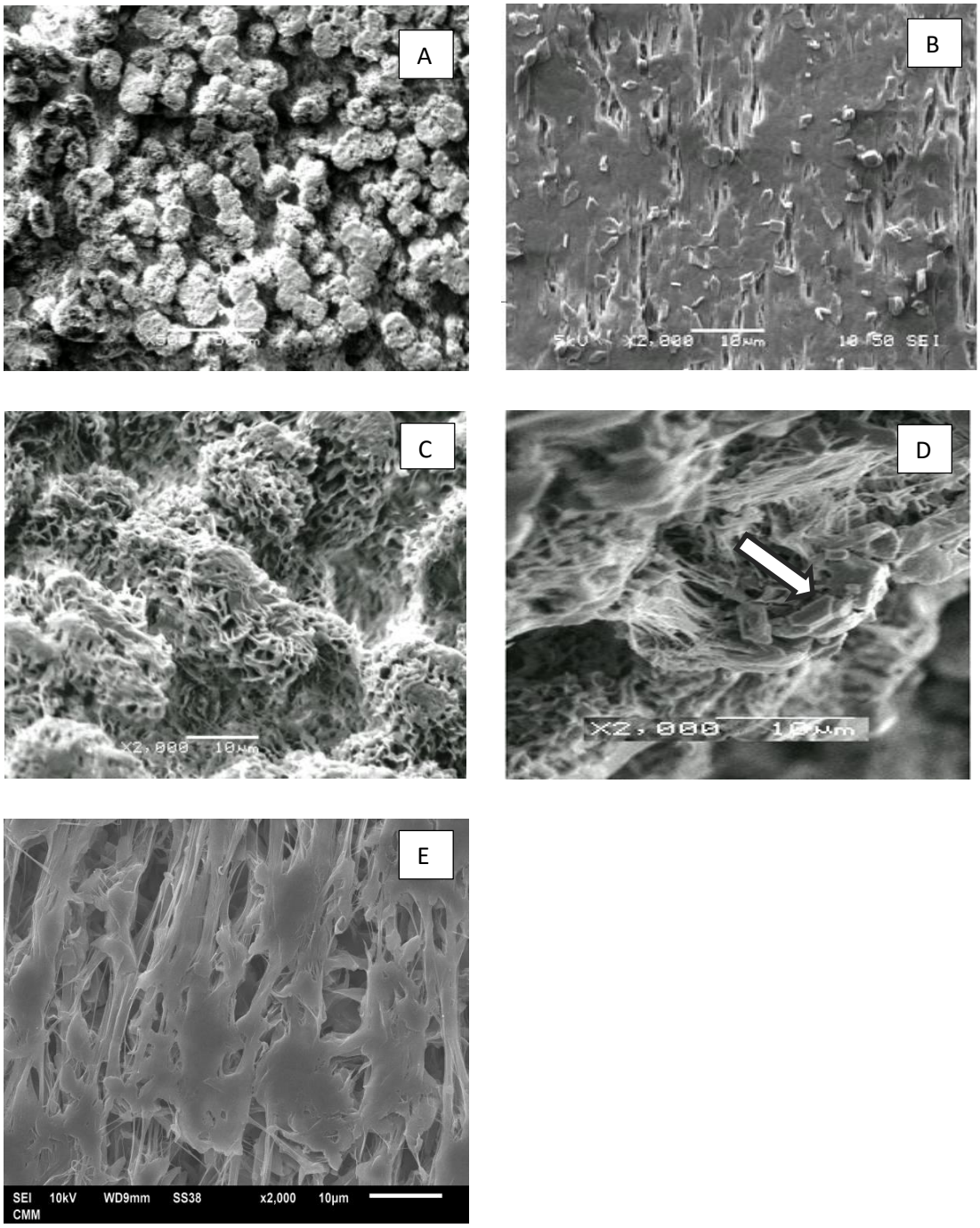

Figure 1: Morphology of drug-free and metronidazole (MTZ)-loaded PCL matrices. A) Interior of drug-free PCL matrix B) Surface of 5.4% MTZ-loaded PCL matrix C) Interior of 5.4% MTZ-loaded PCL matrix D) Interior of 5.4% MTZ-loaded PCL matrix showing presence of drug crystals (arrowed) E) Surface of PCL matrix loaded with 13.7% w/w metronidazole and 10% PEG.

Table 1: Actual loading, loading efficiency and shore hardness of metronidazole in PCL matrices prepared using the rapid cooling technique. Within each column, numbers sharing the same superscript letter are not significantly different (P<0.05).

Theoretical drug loading	Actual loading (%w/w)	Incorporation efficiency	Shore hardness (mN/mm^2)
0	0	0	3986 ± 210^{a}
5	2.0 ± 0.5^{a}	40 ± 10.1^{a}	3841 ± 190^{a}
10	5.4 ± 0.8^{b}	54 ± 8.0^{ab}	1522 ± 75^{b}
15	8.1 ± 0.8^{c}	54 ± 5.3^{ab}	1268 ± 62^{b}
20	10.6 ± 0.7^{d}	53 ± 3.5^{b}	888 ± 32^{c}

In the absence of PEG, loading efficiency of 54% was obtained for the theoretical loading of 15% metronidazole, i.e. only 8.1% loading was achieved (Table 1). Drug loading and loading efficiency improved with increasing PEG loading (Table 2), reaching 13.7% w/w metronidazole loading (91% loading efficiency) when 10% PEG was added. This result is consistent with expectations, as this effect of PEG has been measured previously; for example, loading efficiency of nevirapine increased from 22 to 44% on addition of 8% w/v of PEG as a solid dispersion with the drug to PCL matrices[17]. The reduction in loss of metronidazole with increasing PEG content of the matrix suggests that the PEG phase shields the drug from exposure to ethanol, possibly by forming a viscous solution on dissolution that impedes access of the solvent to the drug particles. PEG may also have dissolved in the ethanol, and so it is likely that there was some loss of PEG during the production. The values given for PEG loading (Table 2) indicate the quantity added, but the actual loading of PEG was not measured in this study.

Table 2: Actual loading, incorporation efficiency and shore hardness of metronidazole (MTZ) in PCL/PEG matrices. 15% w/w metronidazole was added to PCL solution in acetone with the PEG concentration indicated, and the actual quantity of metronidazole loaded was measured using UV spectrophotometry at 319 nm. Within each column, numbers sharing the same superscript letter are not significantly different (P<0.05).

PEG loading (% w/w)	Actual MTZ loading (% w/w)	MTZ Incorporation efficiency	Shore hardness (mN/mm^2)
0	8.1 ± 0.8^{a}	54.0 ± 5.3^{a}	1420 ± 135^{a}
2	8.9 ± 0.7^{ab}	59.3 ± 4.7^{ab}	1322 ± 205^{a}
3	9.9 ± 0.8^{bc}	66.0 ± 5.3^{bc}	1273 ± 165^{a}
4	10.8 ± 0.5^{c}	72.0 ± 3.3^{c}	1155 ± 163^{a}
5	12.5 ± 0.3^{d}	83.3 ± 2.0^{d}	1085 ± 98^{a}
10	13.7 ± 0.3^{d}	91.3 ± 2.0^{d}	653 ± 77^{b}

4.3 Thermal properties

DSC analysis revealed more than doubling of the crystalline content of PCL matrices from around 33% to 76% with increasing metronidazole loading of the material from 0 to 8.1% w/w (Table 3). Previous studies by Chang *et al*. revealed crystallinity levels of 50-75% for drug-free PCL matrices and both increases and decreases in PCL crystallinity depending on the type of drug molecule incorporated in the matrix[18]. Progesterone inclusion (10%) resulted in a major reduction of crystallinity of around 12% from 66% to 54%[18], whereas gentamicin sulphate particulates increased the crystallinity of PCL matrices by 4-8%. Progesterone particulates were considered to inhibit PCL crystal nucleation and growth, while gentamicin sulphate particles acted as nucleating agents to enhance PCL crystallisation. The significant increase in crystallinity of the PCL phase with metronidazole loading measured in the present study indicates the strong effect of the dispersed drug particles on nucleation and crystal growth of PCL. The particles of metronidazole appear to promote heterogeneous or epitaxial crystallisation of PCL, which is known to be influenced

by similarities in the crystal lattice of the substrate and crystallising polymer and also by the surface topography of the substrate (defects, steps and terraces)[31]. The lower crystallinity of the drug-free PCL phase in the present study compared with samples produced by Chang *et al.*[18] reflects rapid cooling of the PCL solution compared with room temperature precipitation technique. Rapid cooling restricts polymer chain mobility and crystal growth. The glass transition temperature indicates the reversible change in the amorphous regions of a polymer from a hard and relatively brittle condition to a viscous or rubbery state. The increase in Tg found with increasing metronidazole loading (Table 3) indicates an interference or restriction of PCL chain mobility due to the presence of the dispersed drug particles in the matrix.

Table 3: Thermal analysis of metronidazole-loaded PCL matrices. Within each column, numbers sharing the same superscript letter are not significantly different (P<0.05).

Theoretical drug loading (%w/w)	Actual Drug loading (%w/w)	Crystallinity (%)	Glass transition temperature (T_g) (°C)
0	0	33.4 ± 2.3^{a}	-60.1 ± 0.6^{a}
5	2.9	42.4 ± 1.9^{b}	-58.1 ± 0.4^{b}
10	5.4	56.1 ± 2.2^{c}	-56.5 ± 0.4^{c}
15	8.1	74.8 ± 1.6^{d}	-55.6 ± 0.5^{cd}
20	10.6	75.6 ± 1.2^{d}	-54.5 ± 0.6^{d}

Thermograms of pure PEG 1500, pure metronidazole and pure PCL matrix show a melting peak at around 52°C, 162°C and 62°C respectively (Figure 2a-c). DSC thermograms of PCL/PEG matrices having a low 3% PEG and containing 9.9% w/w metronidazole loading revealed a main endothermic peak around 67°C with a shoulder at approximately 62°C (Figure 2d) representing melting of the PCL/PEG and PCL mixed phases of the matrix respectively. A higher PEG loading of 10% showed dominance of the PEG melting peak at 58°C with a second melting peak at 62°C corresponding to PCL (Figure 2d) which suggests that PCL and PEG exist in two different phases at higher PEG concentration.

Similar separation of peaks in the DSC thermograph for PEG and the polymer phase was reported previously for PEG-modified polylactic acid films[32] and etanidazole-loaded PLGA/PDLA microspheres[33].

4.4 Shore hardness

The shore hardness values determined by texture analysis for metronidazole-loaded PCL matrices are shown in Table 1. Matrices containing low drug loadings (2% w/w) exhibited similar hardness to unloaded samples (3900 mN/mm^2). Matrix hardness decreased to around 900 mN/mm^2 when the drug loading was increased to 10.6% metronidazole, indicating that excessive drug loading causes deterioration and weakening of the matrix structure, probably by micro-cracking effects which are also influential in relation to drug release behaviour[34]. The hardness of the poly(ethylene vinyl acetate) IVR (Nuvaring®) was found to be 9280 mN/mm^2 which is almost 2.5 times more than the 2% metronidazole-loaded PCL samples. Thus microporous PCL IVRs potentially offer scope for improving user comfort compared with conventional materials. This requires careful consideration when optimising the properties of intravaginal ring devices based on metronidazole-loaded PCL matrices since adequate mechanical properties are required to withstand the flexural loads experienced during insertion and during device residence in the vagina and thus ensure successful clinical performance.

Hardness of the matrices was reduced even further with the addition of PEG. Hardness reduced by more than 50% from 1420 mN/mm^2 to 653 mN/mm^2 when PEG was incorporated at a level of 10% in PCL matrices (Table 2). Increasing the loading of PEG was observed to cause the matrices to be more brittle in nature. This may be explained by PEG interfering with the internal structure of PCL and leading to weakening of the PCL structure. The morphology of PLGA/PDLA polymer microspheres changed even on the addition of only 1% PEG, which was hypothesised to be due to the fact that PEG interacted with the PLGA/PDLA polymer and increased its chain mobility, thereby weakening the polymeric structure[33].

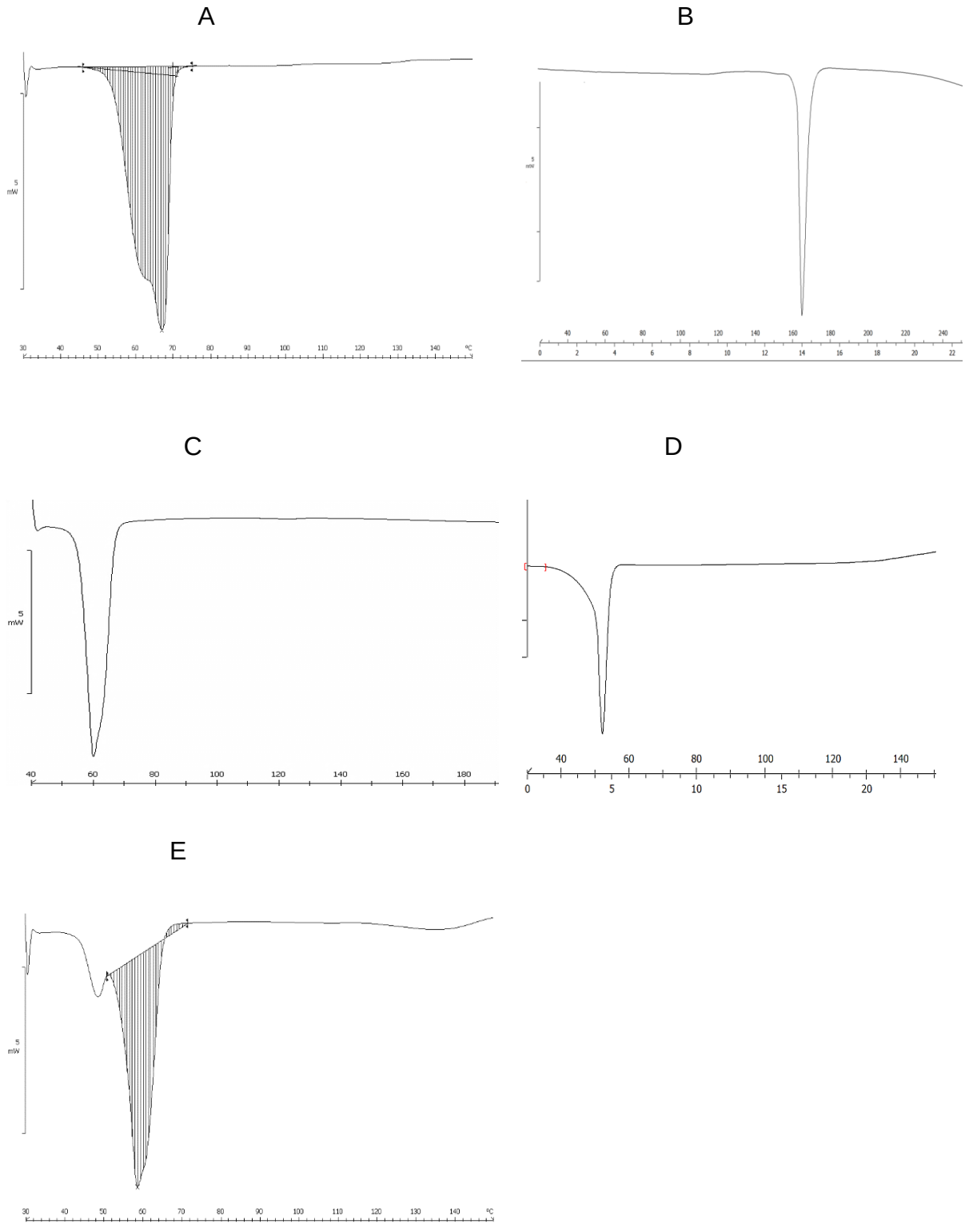

Figure 2: DSC thermogram of A) pure PEG 1500, B) pure metronidazole, C) drug-free PCL matrix, D) 3% PEG with 9.9% w/w metronidazole and E) 10% PEG with 13.7% w/w metronidazole

4.5 *In vitro* release of metronidazole from PCL matrices

Drug release from PCL matrices featuring dispersed drug particles is governed by a number of factors including drug loading, uptake of release medium and drug solubility in the fluid phase, matrix porosity and the rate of drug diffusion in fluid-filled pores of the material. Since PCL exhibits a bioresorption time in excess of two years, the detailed pore structure (pore size, connectivity and tortuosity) can exert a major influence on drug transport from the matrix.

There was a large burst release, measured as the drug concentration in the simulated vaginal fluid after 1 day of immersion of each PCL cylinder (Figures 3 and 4). The magnitude of the burst release increased with drug loading of the matrix from 35% for 2.0% loaded matrices to almost 60% for the most highly loaded PCL matrix (10.6%), with a linear relationship between day 1 release and drug loading (Figure 5; R^2=0.9904). This burst release suggests the presence of large amounts of drug particles at or close to the matrix surface and is supported by the SEM observations described above (Figure 1b). Gradual drug release occurred from day 2 onwards, giving rise to an almost linear profile over the following 12 days that showed little difference between drug loadings, suggesting that drug loading within the core of the PCL was similar for all drug loadings used. In the case of low drug loading, gradual release of metronidazole is expected to occur predominantly through interconnected pores and channels inherent in the microporous PCL matrix since the separation of drug particles will not favour formation of interconnected macropores by dissolution of contacting drug particles. Increasing numbers of interconnected macropores, fissures and channels are expected in the highly loaded systems, due to contact of drug particles and micro-cracking effects[34], which facilitate entry of release medium and enhance drug dissolution and extraction. Around 75% of the drug load was released from all samples by day 13, with no significant difference associated with drug loading, demonstrating in general high pore interconnectivity and thus delivery efficiency. Standard deviation around each mean was reasonably large (Figure 3), which might be due to the sedimentation of the drug during production leading to unequal distribution of drug within the matrices.

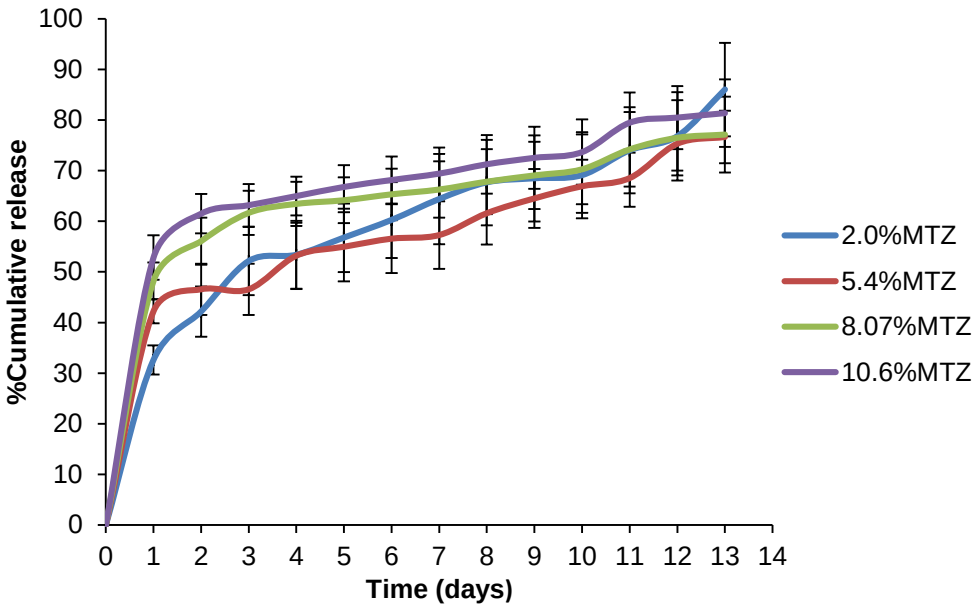

Figure 3: Cumulative (%, mean ± sd of 3 replicates) release of metronidazole (MTZ) from PCL matrices containing 2, 5.4, 8.07 and 10.6% MTZ in simulated vaginal fluid at 37°C

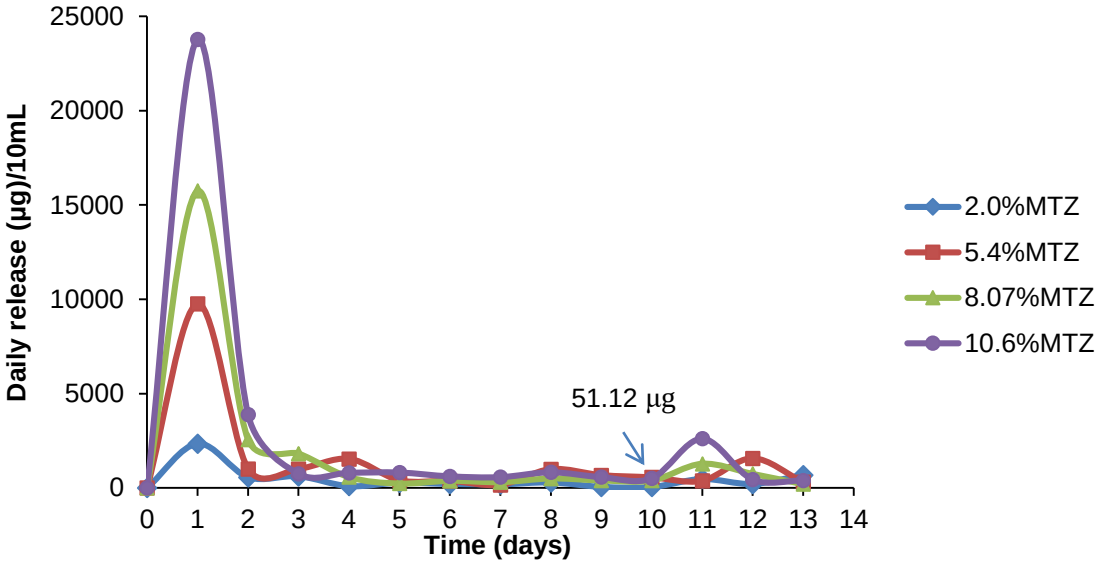

Figure 4: Daily release of metronidazole (MTZ) release (µg) from PCL matrices in simulated vaginal fluid at 37°C (mean of 3 replicates)

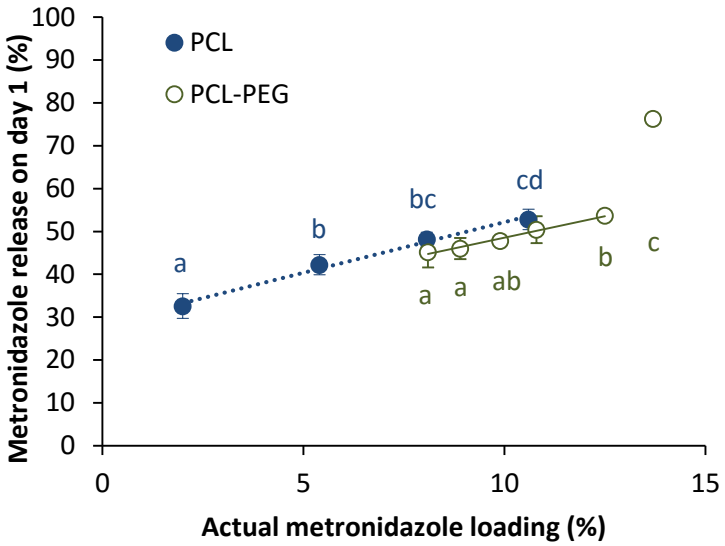

Figure 5: Amount of metronidazole release (%) on day 1 of immersion of PCL matrices in simulated vaginal fluid at 37°C (mean ± sd of 3 replicates) plotted against the metronidazole loading (%) within each of the PCL matrices. Linear regression is shown for PCL matrices (y = 2.36x + 28.59; R^2 = 0.9904), and for PCL-PEG matrices excluding the highest metronidazole loading (y = 2.01x + 28.44; R^2 = 0.9857). For the points on each line analysed separately, points with the same lowercase letter shown above (PCL matrices) or below (PCL-PEG matrices) are not significantly different.

The daily drug release profile (Figure 4) is important because it helps to predict that the amount of drug release is sufficient against the microorganism responsible for the disease during the whole release period. The minimum amount of metronidazole released in a single day was 51 µg measured at day 10 for the 5.4% metronidazole-loaded matrices.

Incorporating PEG into PCL matrices at levels up to 5% was also characterised by a prominent burst effect at day 1 resulting in rapid loss of 45-55% of the metronidazole content (Figure 6). There was a linear relationship between day 1 release and drug loading for matrices prepared using 0 to 5% PEG (Figure 5; R^2=0.9857) but the day 1 burst effect was significantly higher and did not follow the same linear relationship once the concentration of PEG added to the mixture increased to 10%. Excluding this point, at day 1 the concentration of metronidazole released from matrices that incorporated PEG were very similar to those without PEG for equivalent drug loadings (Figure 5) suggesting that PEG had no effect at this early stage of release. In contrast, PCL matrices prepared with 10% PEG may have experienced rapid dissolution of the hydrophilic PEG phase which facilitated drug release from the matrix interior.

The cumulative amount of metronidazole released from PCL-PEG matrices increased gradually over the subsequent 11 days to reach 70-85% for PCL matrices containing up to 5% PEG and 12.5% metronidazole. This behaviour may be attributed to development of an interconnected pores and channels structure following PEG dissolution and extraction within the inherently microporous PCL phase, which facilitates metronidazole transport from the matrix core. Notably, incorporating 10% PEG resulted in release exceeding 90% by day 2 and quickly reaching 95% (Figure 6), indicating rapid production of an extensive interconnected pore network that facilitates ingress of medium, drug dissolution and transport from the matrix. This data indicates that drug release suitable for the 7 day duration of therapy required for metronidazole may be possible by further adjusting the amount of PEG blended with PCL. Similar increase in drug release was noticed on addition of PEG in bi/tri-layered PCL films loaded with a combination of paclitaxel and 5-fluorouracil accelerated paclitaxel release from 60 to 90% within 100 days in PBS when 10% PEG was incorporated[35]. Similarly, 5-fluorouracil release from PCL films was complete within 22 h for films containing 40% drug with 5% PEG, but within only 12 h for films incorporating 40% drug and 15% PEG[36]. The minimum release measured was 61 µg/mL on day 11 for matrices prepared with 4% PEG and containing 10.8% metronidazole (Figure 7) except for the matrices loaded with 13.7% metronidazole and 10% PEG where all the drug released within 5 days.

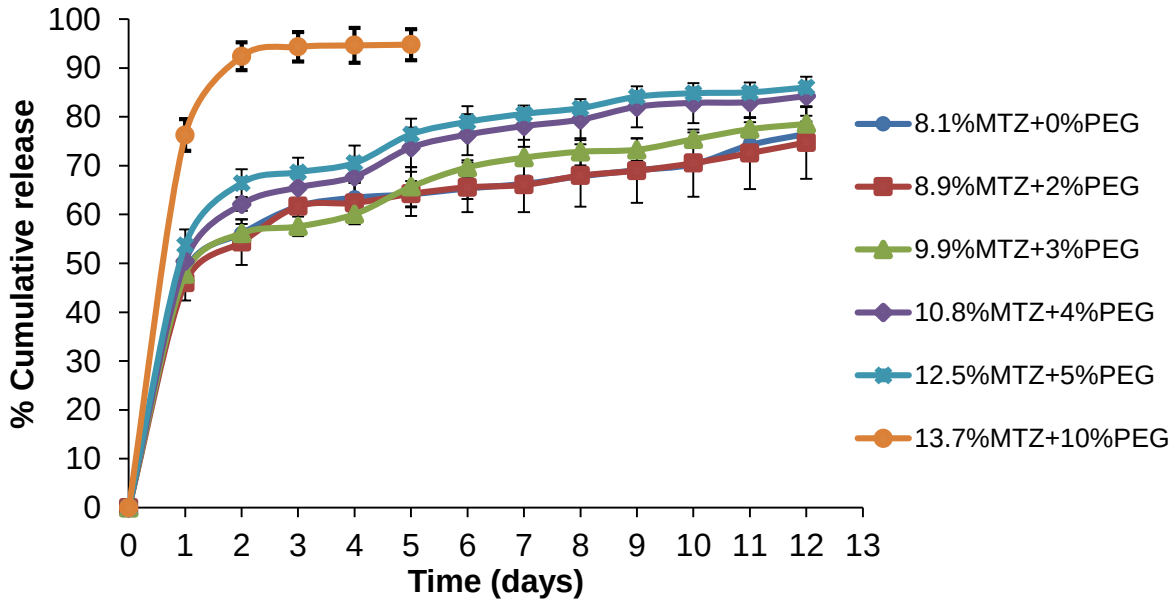

Figure 6: Cumulative release (% of total incorporated) of metronidazole (MTZ) in simulated vaginal fluid at 37°C from PCL matrices containing 0 to 10% PEG

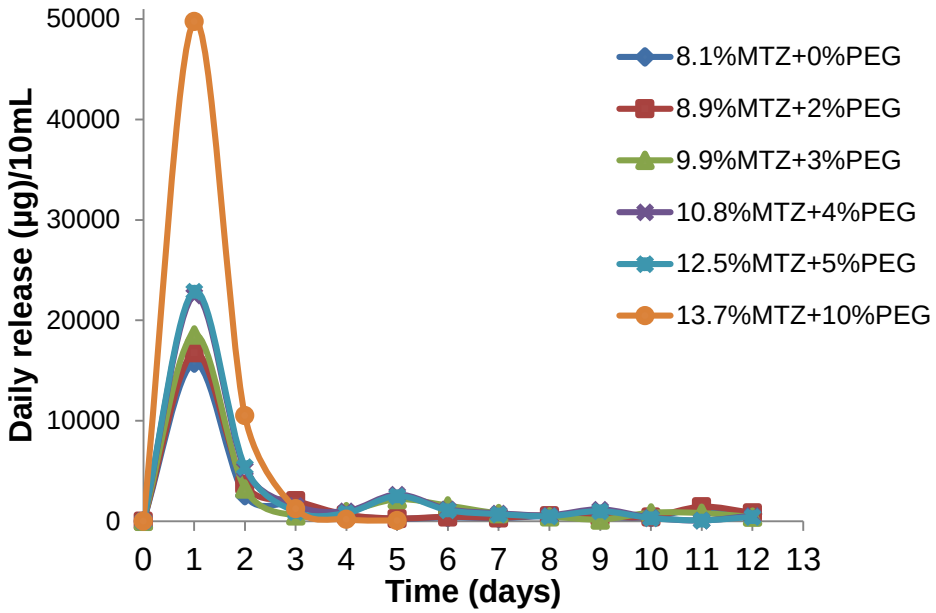

Figure 7: Daily release of metronidazole (MTZ) release (µg per day) in simulated vaginal fluid at 37°C from PCL matrices containing 0 to 10% PEG

4.6 Drug distribution

The Raman spectra of PCL showed a characteristic peak in the region of 3000 cm^{-1} due to C-H stretching; small peaks at around 1800-1700 cm^{-1} are assigned to C=O stretching, while the peak at 1500 cm^{-1} is attributed to δCH_2 and that at 1200-1280 cm^{-1} is due to ΩCH_2[37]. The spectra of PCL matrices revealed an absence of those molecules characteristic of the solvents used (acetone, ethanol) in matrix preparation.

Raman peaks for metronidazole at 1500-1650 cm^{-1} are due to C=N stretching. In-plane and out-of-plane deformation vibrations are normally observed as sharp but weak to medium intensity bands in the region 1300-750 cm^{-1}. Small peaks around 800 cm^{-1} are due to NO_2 group scissoring, wagging, rocking and twisting[38].

The Raman spectra in Figure 8 were generated at radial positions starting from the centre of a 5.4% metronidazole-loaded PCL matrix disk and moving toward the edge. The distinct peaks in the region of 3000 cm^{-1} and 1000-1500 cm^{-1} are due to the presence of the polymer and drug respectively. The traces clearly indicate the change in peak intensity corresponding to differences in drug concentration at different positions within the sample, which reflects the dispersion of drug powder within the PCL matrix. The high intensity Raman spectra obtained at points near the sample edge for the 5.4% metronidazole-loaded PCL matrix (Figure 8) are indicated by the arrow. The high concentration of the

metronidazole was further confirmed by running the RAMAN along the edges (Figure 9). The high intensity peaks represent the presence of high concentration of metronidazole, which support the SEM images of drug crystals located at the sample surface and explain the high burst release behaviour of metronidazole at day 1 of release testing (Figure 4).

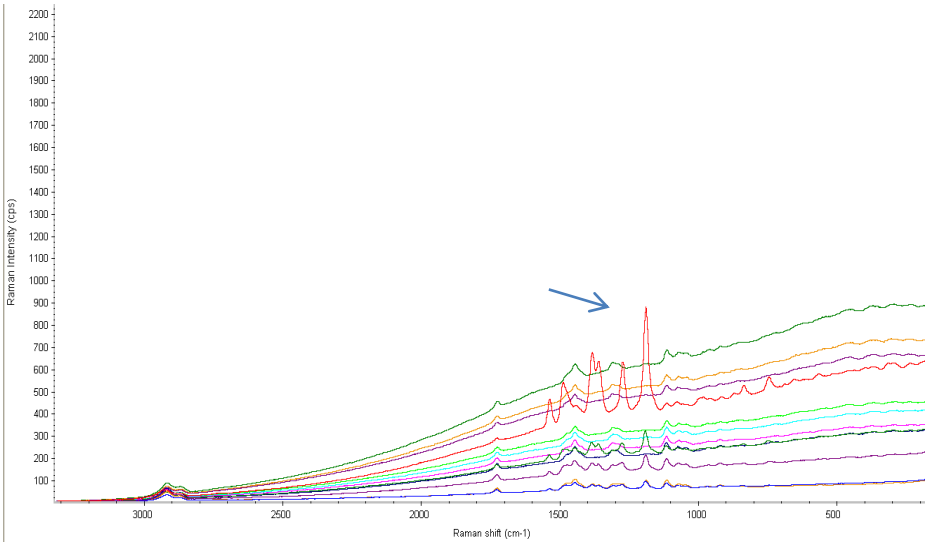

Figure 8: Raman spectra obtained at different radial positions in a transverse section of 5.4% w/w metronidazole-loaded PCL matrix with the spectra displaced on the y-axis to allow the data to be visualised. The arrow indicates high intensity peaks obtained at points close to the edge of the PCL matrix.

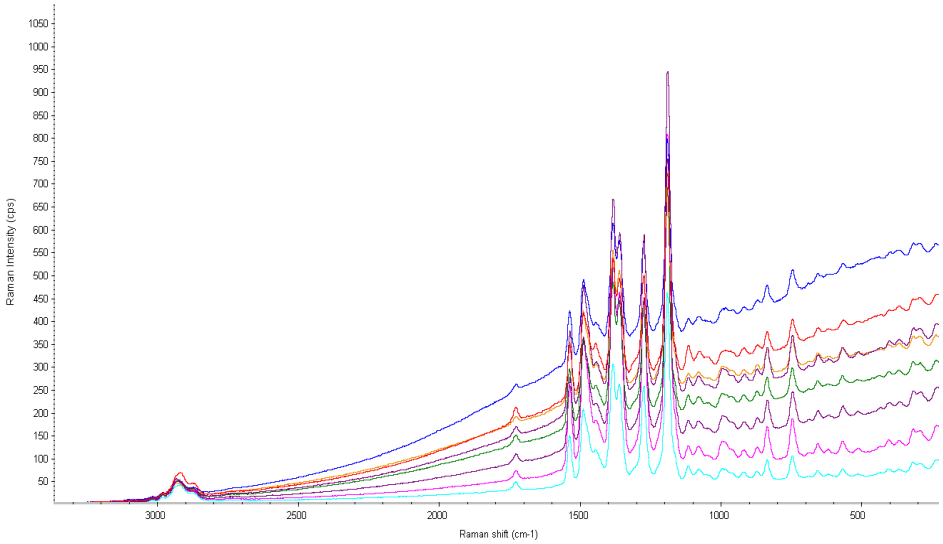

Figure 9: Raman spectra obtained at different edge points in a transverse section of 5.4% metronidazole-loaded PCL matrix, with the spectra displaced on the y-axis to allow the data to be visualised.

4.7 Antibacterial testing

G. vaginalis is an anaerobic, β-haemolytic, oxidase-negative, catalase-negative, gram variable bacterium, which is detected in all women diagnosed with BV and plays an important role in its pathogenesis[5]. The antibacterial activity of non-formulated metronidazole and drug released from PCL matrices into SVF against *G. vaginalis* was investigated using a disc diffusion assay to determine the effect of matrix formulation and matrix residence time in SVF on drug activity and to assess any implications for *in vivo* performance and dosing regimens. A zone of inhibition increasing from 7 to 20 mm diameter was observed for control drug solutions in SVF with increasing concentration from 25-125 µg/ml (Figure 10). No zone of inhibition was observed in the case of control samples comprising SVF alone or release media obtained from drug-free PCL matrices. A linear relationship was observed between metronidazole concentration and the diameter of the zone of inhibition, with a high correlation coefficient (R^2 = 0.962) for the standard.

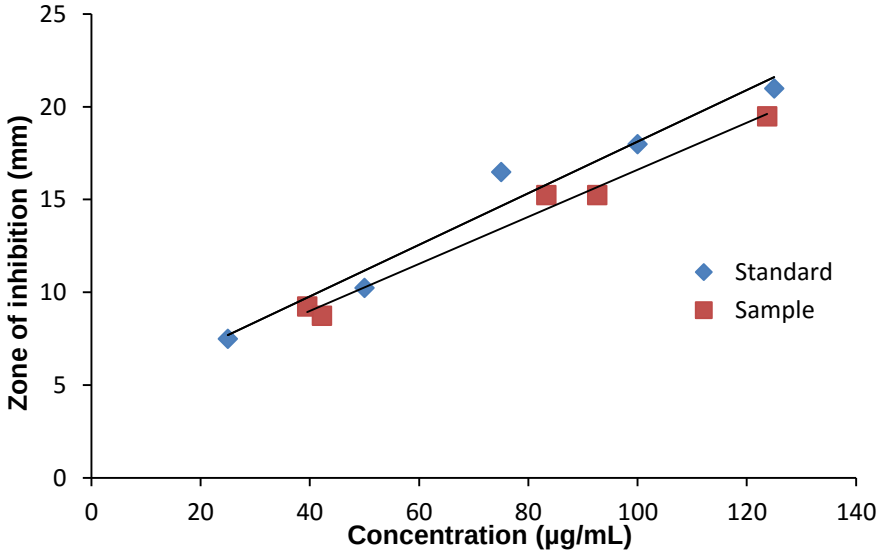

Figure 10: Relationship between concentration of metronidazole and diameter of zone of inhibition against *G. vaginalis* for non-formulated metronidazole (standard) and drug released from PCL matrices into SVF (sample)

The relative antibacterial activity (%) of metronidazole released from 5.4% drug-loaded PCL matrices into SVF was obtained by comparing the diameter of the zone of inhibition obtained for standard metronidazole solution and metronidazole-containing release media at equivalent drug concentrations. A high relative antibacterial activity (88-97%) was exhibited by released drug over a 10 day release period (Figure 11). No significant difference in activity was noticed, indicating that the drug is stable during the study period.

The slightly lower activity in comparison to pure drug might be due to prolonged exposure of the drug within the PCL matrix to elevated temperature (37°C for up to 10 days) and the fairly complex biochemical environment presented by the SVF.

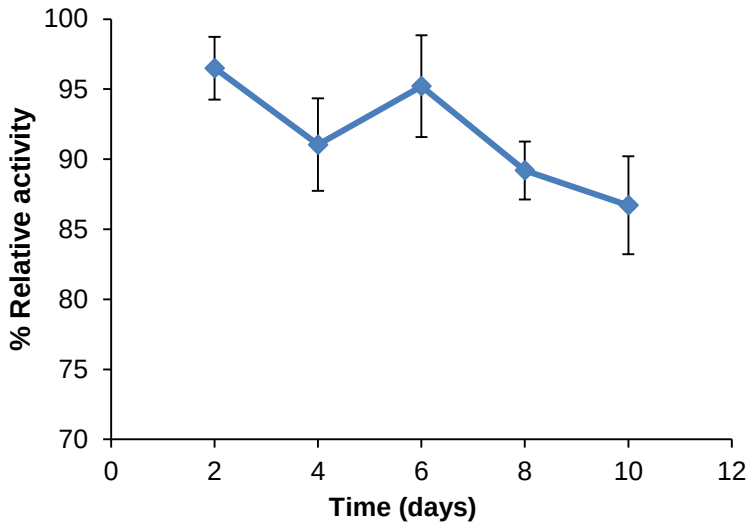

Figure 11: Relative activity (%) of metronidazole released from 5.4% drug-loaded PCL matrices in simulated vaginal fluid at 37°C over 10 days

A similar relative antibacterial activity of 86-96% was measured for metronidazole released from the PCL/PEG matrices compared with metronidazole solutions in SVF of equivalent concentration, indicating that PEG has no effect on the antibacterial property of the drug.

The minimum daily amount of metronidazole released into 10 mL SVF from PCL matrices was 51 µg at day 10 from a 5.4%-drug loaded matrix (Figure 4). For matrices that also incorporated PEG, the minimum was 61 µg/mL on day 11 for 4% PEG and containing 10.8% metronidazole; this excludes the matrices containing 10% PEG and 13.7% metronidazole which had complete release within 5 days (Figure 6). If an IVR of linear length 150 mm (outer diameter 58 mm, inner diameter 38 mm) and weight (1.5 g) were produced, it would be approximately 3.5 times that of the matrices studied herein (45 mm, 0.4 g). Therefore the minimum release amount of 5.1 µg/mL/day corresponds to a drug release rate from a PCL IVR of around 18 µg/mL/day. Indeed, in a concurrent PhD project, nevirapine and tenofovir release from PCL matrices and PCL IVRs was directly proportional to PCL length[40]. The predicted concentrations of drug which would be released from a PCL IVR into vaginal fluid are above the minimum inhibitory concentration (MIC) against *G. vaginalis* (2-12.8 µg/mL)[39] during the study period. This is based on the assumption that the *in vitro* release rate from PCL matrices is similar to the *in vivo* release rate from a PCL vaginal ring and a maximum vaginal fluid turnover rate of 8 mL/day

applies. These estimates do not take into account the complex variations in vaginal fluid volume and biochemical composition over time, or the possibility of systemic uptake of drug.

5. Conclusion

Metronidazole antibacterial activity was retained after loading and releasing from PCL matrices. The amount of drug released into SVF on each day of the 14 day study was calculated to be more than the MIC of the bacteria causing bacterial vaginosis.

Loading efficiency was only a maximum of 54%. Addition of PEG into the mixture during matrix formation raised incorporation efficiency considerably, for example addition of 10% PEG was associated with 91% of the metronidazole being incorporated. Unfortunately around 95% of drug came out within 5 days but a small reduction in PEG concentration would be expected to provide a maximum release over a 7 day period, which is currently the duration of therapy used. The daily drug release from all the matrices studied, loaded with 0 – 5% of PEG and 8.1–12.5% metronidazole, was more than the MIC against an organism which is major cause for bacterial vaginosis, *G. vaginalis*.

As PCL matrices show some potential for the intravaginal delivery of antibacterial agents that treat STIs, the next step must be to determine whether they are safe for vaginal use. Additionally, although PEG improved drug loading, it also lead to the deterioration of mechanical properties of the polymer so subsequent research should focus on improving drug loading by modification of the method but without the addition of PEG.

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CHAPTER 3

INVESTIGATION OF POLYCAPROLACTONE MATRICES FOR THE INTRAVAGINAL DELIVERY OF DOXYCYCLINE

1. Abstract

Poly(ε-caprolactone) (PCL) matrices loaded with doxycycline were produced by rapidly cooling suspensions of the drug powder in PCL solution in acetone. Drug loadings of 5, 10 and 15% w/w of the PCL content were achieved. Exposure of doxycycline powder to matrix processing conditions in the absence of PCL revealed an endothermic peak at 65°C with the main peak at 167°C, suggesting solvatomorph formation. Rapid 'burst release' of 24% to 32% was measured within 24 h when matrices were immersed in simulated vaginal fluid (SVF) at 37°C, due to the presence of drug at or close to the matrix surface; which is further confirmed by scanning electron microscopy. Gradual release of 66-76% of the drug content occurred over the following 14 days. SVF containing doxycycline released from drug-loaded PCL matrices retained 81-90% antimicrobial activity compared to the non-formulated drug. The concentrations of doxycycline predicted to be released into vaginal fluid from a PCL matrix in the form of an intra-vaginal ring (IVR) would be sufficient to kill *N. gonorrhoea* and many other pathogens. These results indicate that PCL may be a suitable polymer for controlled intra-vaginal delivery of doxycycline for the treatment of STIs.). No significant reduction in cell viability was recorded when leachates prepared by incubating PCL matrices in SVF for 28 days were tested against vaginal cell line (Vk2/E6E7) indicating PCL as a favourable polymer for production of vaginal delivery devices.

2. Introduction

Around 500 million cases of four of the major curable sexually transmitted infections (STIs), *Chlamydia*, gonorrhoea, syphilis and trichomoniasis, were recorded worldwide in 2008, an increase of 11% from 2005[1], confirming the urgent need for improved prevention and treatment strategies. Doxycycline belongs to the tetracycline group of antibiotics and is highly effective against Gram-positive, Gram-negative, aerobic and anaerobic bacteria, *Mycoplasma*, *Chlamydia trachomatis* and some protozoa[2]. Thus, doxycycline is a commonly prescribed medication for the treatment of STIs. It exerts a bacteriostatic action by inhibiting bacterial ribosome function and therefore protein biosynthesis, while antiprotozoal activity results from inhibition of apicoplast ribosomal subunits leading to impaired fatty acid synthesis[3]. Doxycycline 100 mg given orally twice a day for 7 days is recommended by the Centre for Disease Control and Prevention for the treatment of genital Chlamydial infection[4] and has been prescribed in combination with cephalosporin for the treatment of gonococcal infection[5]. Extended treatment, involving oral dosing (100

mg twice daily) for 14 days is recommended for the treatment of pelvic inflammatory disease[6] and a dose of 100 mg twice daily for 21 days is first line treatment of lymphogranuloma venereum[7]. Doxycycline may also be prescribed as a second line of treatment for syphilis when patients are allergic to penicillin[8].

Doxycycline, being a lipophilic drug shows excellent tissue penetration and access to sites of infection in the female genital tract following oral administration and systemic distribution[9]. However, oral dosing can cause gastrointestinal side effects such as nausea, vomiting, diarrhoea, epigastric burning and esophageal ulcers[10,11]. In addition, the long duration of treatment may result in non-compliance issues, whereby patients do not complete the course of medication, resulting in an increased probability of treatment failure and drug resistance[12].

Vaginal formulations, including tablets, gels and creams, have been widely investigated as alternatives to oral dosage forms since they avoid first pass metabolism, gastrointestinal and wider systemic exposure to drug molecules which can produce severe side effects. In particular vaginal formulations of microbicides are receiving increasing attention for the treatment and prevention of STIs, especially HIV/AIDS. However, conventional semisolid formulations suffer major drawbacks of leakage and they are 'messy' to apply and there are concerns over inadequate coverage of the vaginal epithelium[13]. These disadvantages have been avoided by the development of intra-vaginal ring (IVR) devices which facilitate insertion and retention and allow long-term drug release in a predictable and controlled manner, thereby increasing patient acceptability[14,15] and reducing compliance problems[16,17].

IVRs are conventionally manufactured from silicone elastomer, thermoplastic polyurethane and polyethylene vinyl acetate (pEVA). However, these polymers require high processing temperatures (silicone elastomer 80°C, pEVA 140°C) and doxycycline is prone to thermal degradation[18-20]. PCL could be a promising option for the delivery of doxycycline, due to the lower melting temperature of 60°C. PCL is synthetic polyester that has been studied extensively for controlled drug delivery and tissue engineering applications in a wide variety of forms including films, fibres, micro- and nanoparticles[21]. Microporous matrices of PCL have been investigated previously for vaginal delivery of the antivirals nevirapine and tenofovir[22,23], the antibacterials gentamicin and metronidazole[24,25], and the antifungal miconazole[26], for prevention and treatment of STIs. The aim of this study was to explore the potential of PCL matrices for vaginal delivery of doxycycline through investigations of

the preparation, morphology, thermal and compressive properties and *in vitro* drug release of doxycycline from PCL matrices. In the previous study, the drug loading was very less which means the drug lost during production step. In the current study omission of drug step was tried to improve the drug loading to 100%.

3. Materials and methods

3.1 Materials

Polycaprolactone (MW 115,000 Da, Capa 650) was obtained from Solvay Interox (Warrington, UK) and doxycycline hyclate from Alfa Aesar (Ward Hill, MA, USA). Sodium chloride, potassium hydroxide, calcium chloride, bovine serum albumin, glucose, glycerol, urea, lactic acid and acetic acid were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Solvents (acetone, acetonitrile) were of analytical grade. CO_2 generating kit sachets were obtained from BD (New Jersey, USA). Thayer Martin agar, Lactobacilli MRS agar plates and anaerobic sachets were purchased from Micromedia (Melbourne, VIC, Australia)**.**

3.2 Preparation of doxycycline loaded PCL matrices

A slight modification of a previously reported method[25] was used to produce the matrices. A 15% w/v solution of polycaprolactone in acetone was produced by heating at 45°C for 45 min. Doxycycline hyclate powder was added in concentrations of 5, 10 and 15% w/w of the PCL content, and the resulting suspension of drug in PCL solution was poured into polypropylene syringes (3 mL) and rapidly cooled at -80°C to produce a cylindrical polymeric matrix loaded with drug. After the cooling phase at -80°C for 24 h polymer matrices were removed from the syringes and dried for 24 h under ambient conditions to evaporate solvents. The resulting cylindrical matrices were loaded with 5% w/w, 10% w/w and 15% w/w doxycycline hyclate and had an average weight of 478 ± 6.3 mg, a diameter of 6.5 ± 0.5 mm and length 45.0 ± 5 mm.

3.3 Morphology of drug loaded PCL matrices

Scanning electron microscopy (SEM) (JEOL-6610LV SEM, Jeol Ltd., Tokyo, Japan) was performed on the drug-free and drug-loaded PCL matrices to investigate the internal and surface morphology. Samples for SEM were mounted on aluminium stubs using adhesive carbon tabs and sputter-coated with platinum using an Eiko sputter-coater prior to examination at 10 KV.

3.4 Thermal properties of doxycycline-loaded PCL matrices

The effect of drug loading on the thermal characteristics of PCL was investigated using differential scanning calorimetry (DSC; DSC 1 STARe System, Mettler-Toledo, Ltd., Melbourne, VIC, Australia) under a nitrogen atmosphere. Samples of drug-free and doxycycline-loaded PCL matrices were cut, weighed and sealed in aluminium pans and subsequently heated from 30 to 330°C at 10°C/min. The crystallinity of the PCL phase was obtained using the DSC software (STARe) based on the heat of fusion value of 139.5 J/g for fully crystalline PCL polymer[24].

A DSC analysis was carried out of doxycycline powder that had been subjected to the same conditions used for matrix production but in the absence of PCL to investigate possible changes in drug crystallinity in the matrix caused by process conditions. This approach was adopted to compensate for the low weight of doxycycline in the PCL matrix samples which results in low enthalpy changes and therefore difficulties in obtaining information about drug crystallinity. Thus a weight of doxycycline powder corresponding to that used for matrix production was dispersed in acetone at 45°C and held at -80°C for 24h before drying under ambient conditions. Samples of treated and untreated doxycycline were analyzed by DSC as described above.

3.5 Biocompatibility evaluation of PCL matrices

PCL matrices of approximate weight 400 mg (accurately weighed) were immersed in 5 mL of sterile SVF in an incubator (Heidolph Inkubator 1000, Germany) at 37°C with shaking (120 cpm) for 4 weeks. Samples of SVF were collected at specific time points (1, 7, 14, 21 and 28 days) and stored at -5°C until testing.

3.5.1 Biocompatibility with vaginal epithelial cells

The potential toxicity of extractables associated with production of PCL matrices was evaluated *in vitro* using the vaginal epithelial cell line (Vk2/E6E7). The cells were cultured in keratinocyte serum-free media supplemented with 0.1 ng/mL EGF, detached from the flask surface after 10 minutes. A 1:1 mixture of Dulbecco's modified eagle's media and Ham's F12 media containing 10% fetal bovine serum (8 mL) was added to neutralize trypsin. The suspended cells were transferred into a centrifuge tube and spun down for 2 minutes. The supernatant was discarded, the cells were resuspended in fresh serum-free growth media and 3 mL aliquots were transferred into fresh cell culture vessels. After 5 passages, cells were seeded in 96-well plates (20,000 cells/well in 200 µL of growth

media) and incubated for 24 h. The growth media were subsequently replaced with the test and control samples and incubated for an additional 24 h. Prior to testing, the samples were filtered using 0.2 µm syringe filters under sterile conditions and diluted 1:6 with culture media (KSF supplemented with 0.1 ng/mL EGF, 50 µg/mL BPE, 1% v/v P/S, 0.4 mM $CaCl_2$). SVF diluted with nutrient media (1:6 dilutions) were used as no treatment control, SVF as toxic control and nutrient media used as positive controls, respectively. The percentage cell viability in each well following exposure to test samples (SVF which had contained PCL matrix) for 24 h was obtained by comparison with the no treatment control. MTS reagent (10 µL) was added to each well and the plate was incubated for 1 h at 37°C. The absorbance was measured at 490 nm using a Multi-Mode Microplate reader (iMARK™, Bio-Rad laboratory Inc., USA) using 650 nm as the reference wavelength. Data were compared with the control (SVF diluted with culture media, 1:6) using one way ANOVA.

3.5.2 Biocompatibility evaluation with a human vaginal isolate of *Lactobacillus jensenii*

Lactobacilli are an important part of the vaginal flora defense mechanism, helping to maintain an acidic pH and providing competition for epithelial cell binding sites, thus reducing the probability of infection by invading microorganisms[30]. Cytotoxicity testing was performed for PCL matrices using *L. jensenii* (ATCC 25258) which is a human vaginal isolate from a dominant species present in the human vagina. The purpose of the experiment was to determine whether any extractables were present in the PCL matrices that could inhibit growth of *L. jensenii.* Bacteria for use in the assay were cultured in MRS broth at 37°C under anaerobic conditions for 24 h and the culture was subsequently diluted to obtain an optical density of 1 using a spectrophotometer (Lovibond, Tintometer, Dormund) at 550 nm. The resulting suspension of lactobacilli (100 µL) was seeded in cell culture bottles containing 8 mL of MRS broth and 2 mL of SVF media that had contained PCL matrices. Samples were incubated for 24 h prior to determining cell growth by measuring the optical density at 550 nm. SVF incubation media in combination with MRS broth (1:4) served as the positive control and SVF served as the negative control.

3.6 Shore hardness testing of PCL matrices

The shore hardness of drug-free and doxycycline-loaded PCL matrices was measured using a CT3 Texture Analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA). Cylindrical PCL matrices were mounted horizontally and compressed perpendicular

by using a 2 mm diameter, flat-ended, cylindrical probe (TA39) at a speed of 0.1 mm/min to depth of 2.0 mm. The shore hardness was calculated on the basis of force required to compress the matrix at a depth of 2 mm. The same test procedure was carried out on a pEVA IVR (Nuvaring®, Schering-Plough Pty limited, North Ryde, NSW, Australia) for comparison.

3.7 *In vitro* doxycycline release

A drug release study was performed in SVF on previously weighed cylindrical doxycycline-loaded PCL matrices (length 45 mm) to simulate drug delivery from a section of a ring shaped device. Prior to release testing the ends of the matrices were sealed by dipping in 5% w/v solution of PCL in acetone and then dried in air. Triplicate samples were placed separately in 10 mL of SVF and retained at 37°C in a temperature controlled incubator. SVF contained 3.51 g NaCl, 1.40 g KOH, 0.222 g $Ca(OH)_2$, 0.018 g bovine serum albumin, 2.00 g lactic acid, 1.00 g acetic acid, 0.16 g glycerol, 0.4 g urea and 5.0 g glucose in 2 L of distilled water. The pH was adjusted to 4.2 using 10% HCl[27] and then filtered to remove any impurity. Each day the release media were collected and replaced with fresh SVF daily during the 14 days study. The drug concentration in the release media was analysed by using a Shimadzu HPLC equipped with binary pump and SPD-M20A photodiode array detector (50µl injection volume), 2.1 X 150 mm, 5 µm Agilent Extend C-18 (Agilent Technologies, CA, USA). Gradient elution was performed with water (eluent A) and acetonitrile (ACN, eluent B). The gradient was 15% (v/v) methanol in MilliQ water, increasing to 70% at 0.5 mL/min over 10 min, then held for 5 min, with the detection at 276 nm and sample injection volume of 50 µL. Standard curves were generated using calibration samples ranging from 10-60 µg/mL. The amount of doxycycline contained in each sample of SVF release medium was obtained by comparison with the standard curve. Separate samples of release media were stored at 5°C until antibacterial activity of doxycycline released from the matrices has been tested on microorganisms. Blank PCL matrices were used as control and all the experiments were performed in triplicates.

3.8 Assay of antibacterial activity of released doxycycline

The antibacterial activity of doxycycline released from the PCL matrices into SVF was studied using *Neisseria gonorrhoea* using the disc diffusion method (M02-A10) established by the Clinical and Laboratory Standards Institute (950 West Valley Road, Suite 2500 Wayne, PA 19087 USA)[28]. *N. gonorrhoea* was collected from an overnight culture in Heart Infusion broth (Oxoid) and diluted to an optical density of 1.0-1.1 at a wavelength of 600

nm (Lovibond, Tintometer, Dormund). Aliquots (100 µL) of the resulting suspension of *N. gonorrhoea* were spread evenly on Thayer Martin Agar. A blank disc (Oxoid) was placed at the centre of each inoculated plate and 50 µL of SVF containing doxycycline released from the PCL matrices along with standard samples comprising solutions of non-formulated doxycycline in SVF (concentration range 30-150 µg/mL) were added to separate discs. Plates were incubated at 37°C for 24 h under modified atmospheric conditions containing 10% CO_2 prior to measurement of the diameter of the zone of inhibition of test samples and standard (non-formulated) drug samples of the same concentration. SVF and release media that had contained drug-free PCL matrices were included as controls.

3.9 Statistical analysis

Significant difference in glass transition temperature, crystallinity and shore hardness were tested using one-way ANOVA with a Tukey multiple comparisons ($p<0.05$).

4 Results and discussion

Doxycycline-loaded PCL matrices, produced by rapidly cooling suspensions of the drug powder in PCL solution in acetone, present a uniform structure with an absence of cracks and voids in the sample surface and interior. The microporous morphology comprised irregular pore shapes with pore dimensions, generally in the range 1-5µm (Figure 1). The flat areas in Figure 1B and 1C may be formed by contact of polymer with the mould surface during matrix hardening. Large numbers of drug particles are visible on the matrix surface (Figure 1C) and in the matrix interior (Figure 1D). The shape and dimensions (10-100 µm) of the doxycycline particles prior to matrix production are shown in Figure 1A. Comparison with drug-loaded matrices (Figure 1D and 1E) indicates that particulates were reduced in size during dispersion in the PCL solution as part of matrix production.

A significant decrease in crystallinity of the PCL phase of 7% was measured on loading the PCL matrices with 15% w/w doxycycline in comparison with the blank PCL matrices (Table 1). Previous studies of microbicide-loaded PCL matrices have shown that the crystallinity of PCL may increase or decrease with increasing drug loading and the ordered, close-packed crystal lattice will, in turn, influence the transport of release medium and drug diffusion through the matrix. Loading progesterone into PCL at a concentration of 10% also resulted in a decrease in crystallinity from 66% to 54%, possibly caused by inhibition of PCL nucleation and crystal growth by suspended drug particles[29]. In contrast,

metronidazole incorporation in PCL matrices increased the crystallinity of the PCL phase from 33% to 76% as the drug loading increased from 0 to 10.6% w/w[25] and similar behaviour was recorded for gentamicin sulphate-loaded PCL matrices. This was explained in terms of the drug particles acting as nucleating agents for PCL crystal growth[24].

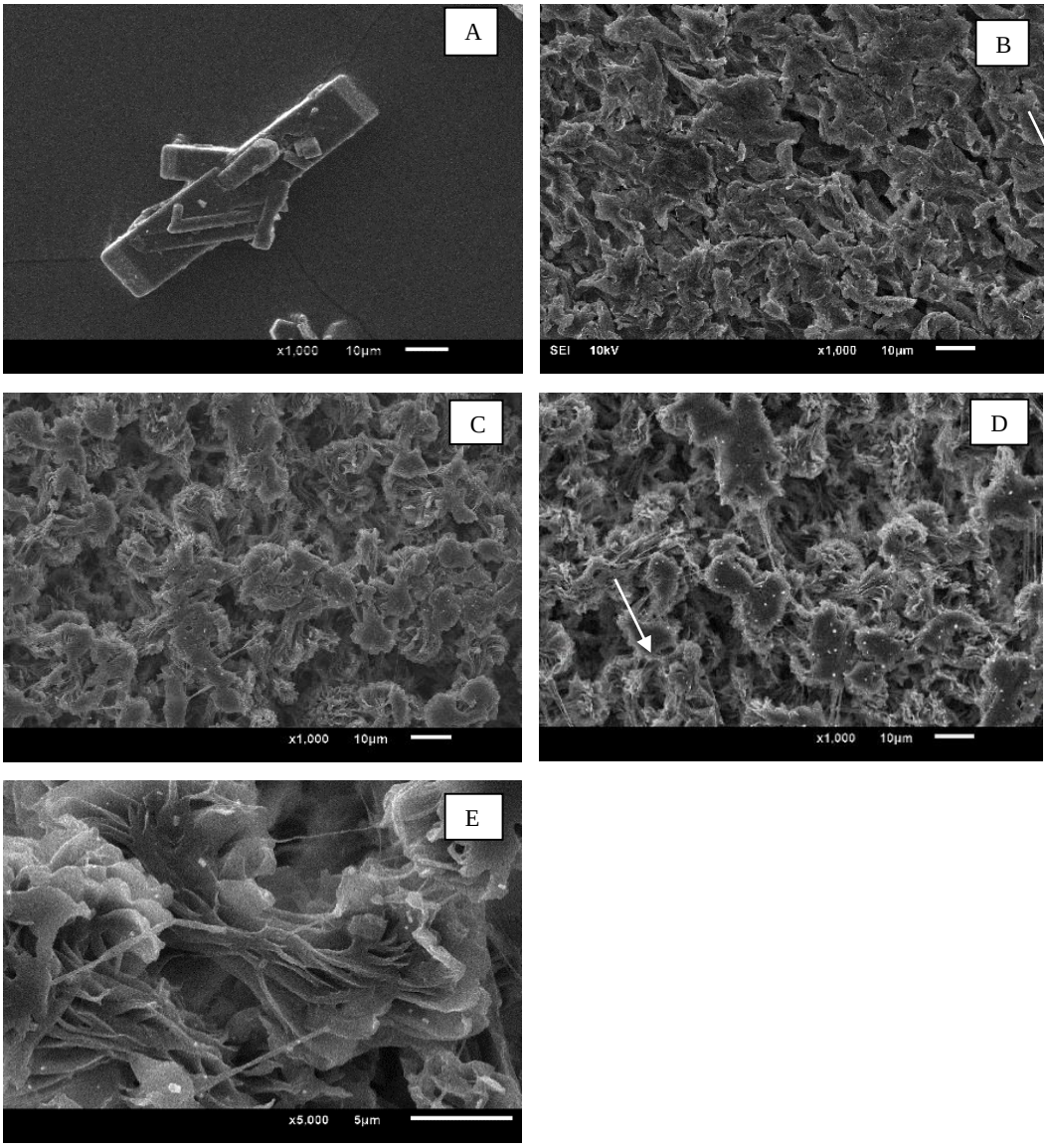

Figure 1: Morphology of PCL matrices revealed by scanning electron microscopy A) Doxycycline powder B) Surface of drug-free PCL matrix C) Surface of 10% doxycycline-loaded PCL matrix D) Interior of 10% doxycycline-loaded matrix E) Interior of 10% doxycycline-loaded matrix at higher magnification

Table 1: Thermal analysis and shore hardness of PCL matrices loaded with doxycycline. Data are the mean ± sd of 3 replicates. Values within columns that have the same superscript letter are not significantly different (p<0.05)

Drug loading (% w/w)	Glass transition temperature (°C)	Crystallinity (%)	Shore hardness (mN/mm^2)
0	64.8 ± 1.5^{a}	80.2 ± 1.9^{a}	1173 ± 15^{a}
5	71.2 ± 1.1^{b}	79.0 ± 1.2^{a}	1558 ± 30^{b}
10	68.9 ± 2.0^{b}	77.0 ± 2.3^{ab}	1758 ± 31^{c}
15	63.3 ± 1.3^{a}	73.8 ± 1.5^{b}	1937 ± 33^{d}

Thermograms obtained for doxycycline (Figure 2A) showed the prominent and typical endothermic peak at around 167°C which has been ascribed to crystal melting and the major exothermic peak at around 220°C due to thermal decomposition[30,31]. Dispersion of doxycycline in acetone followed by retention at -80°C resulted in the emergence of a broad, low endothermic peak at approximately 65°C in combination with the usual peak at 167°C (Figure 2B). In other samples, only a single peak was observed at 65°C with disappearance of the endothermic peak at 167°C (Figure 2C), indicating gradual conversion to the lower melting point derivative. The explanation for this behaviour is unclear at present but gradual formation of a solvatomorph is suggested due to inclusion of acetone molecules in the doxycycline crystal lattice, resulting in a change in crystal structure and unit cell. This finding has implications for therapeutic efficacy due to the effect of different solid forms of a drug on dissolution behaviour for example.

Thermograms of doxycycline-loaded PCL matrices displayed the prominent endothermic, melting peak at 60°C corresponding to melting of the PCL phase (Figure 2D).The melting peak of doxycycline at 167°C was not detected, possibly due to the low drug content of the matrix sample. However, a trace of the major exothermic peak at 220°C was in evidence. Detection of any endothermic peak at 65°C resulting from process-related changes in doxycycline's crystal structure would be masked by the melting peak of PCL.

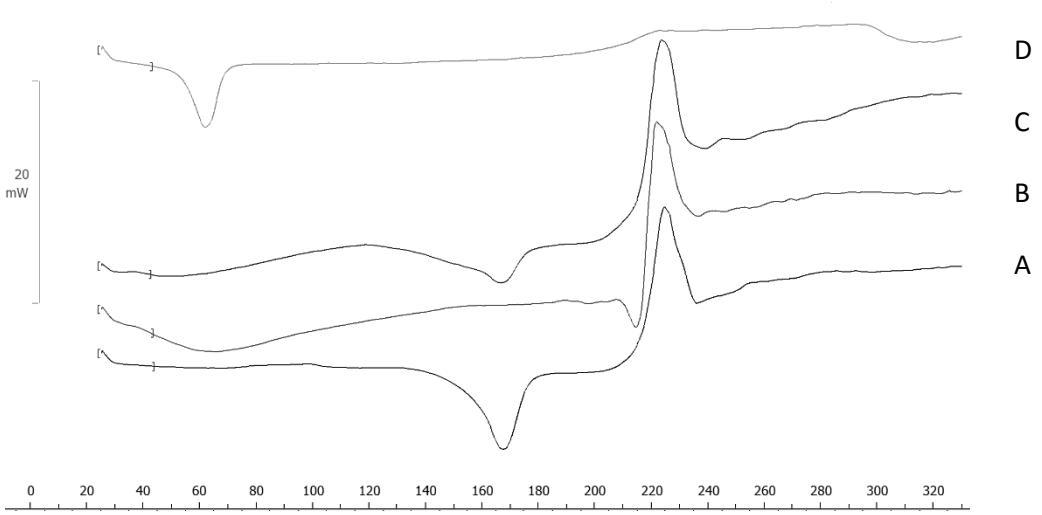

Figure 2: DSC thermogram of A) Untreated doxycycline powder B) Doxycycline powder (drug equivalent to 5% w/w doxycycline loading PCL dispersed in acetone and retained at -80°C) C) Doxycycline powder (drug equivalent to 10% w/w doxycycline loading PCL dispersed in acetone and retained at -80°C) D) 15% doxycycline-loaded PCL matrix.

The favourable biocompatibility of PCL has been widely documented and underpins the extensive investigation of this polymer for drug delivery and tissue engineering applications. For example, cell viability of 95% was recorded when PCL microfibers were assessed using a mouse fibroblast cell line[32], while no significant effect on cell viability occurred when PCL nanoparticles were incubated with mouse embryonic fibroblast cells (NIH 3T3), human cervix carcinoma cells (HeLa) and human osteosarcoma cells (MG63)[33]. Recent studies have utilized the Vk2/E6E7 human vaginal cell line to assess the suitability of topical microbicides and other pharmacological agents intended for vaginal application[34,35]. In the present study Vk2/E6E7 cells were employed to identify possible detrimental effects on the vaginal epithelium due to the release of extractables from the matrix. SVF diluted with culture media 1:6 was used as the control and assigned the nominal value of 100%; all other data were normalized to this value and compared with it. Consequently, exposure of cells to the more favourable environment of culture media alone resulted in greater than 100% viability. SVF alone was found to cause a major reduction in Vk2/E6E7 cell viability due to its low pH of 4.2 and, with nonoxynol-9, acted as a negative control (Figure 3). No significant reduction in cell viability was recorded when samples of drug-free PCL matrix were incubated in SVF for 28 days and the incubation media (diluted 1:6 with culture media) was tested for Vk2/E6E7 cell viability (Figure 3)

indicating the favourable biocompatibility of PCL matrices for production of vaginal delivery devices.

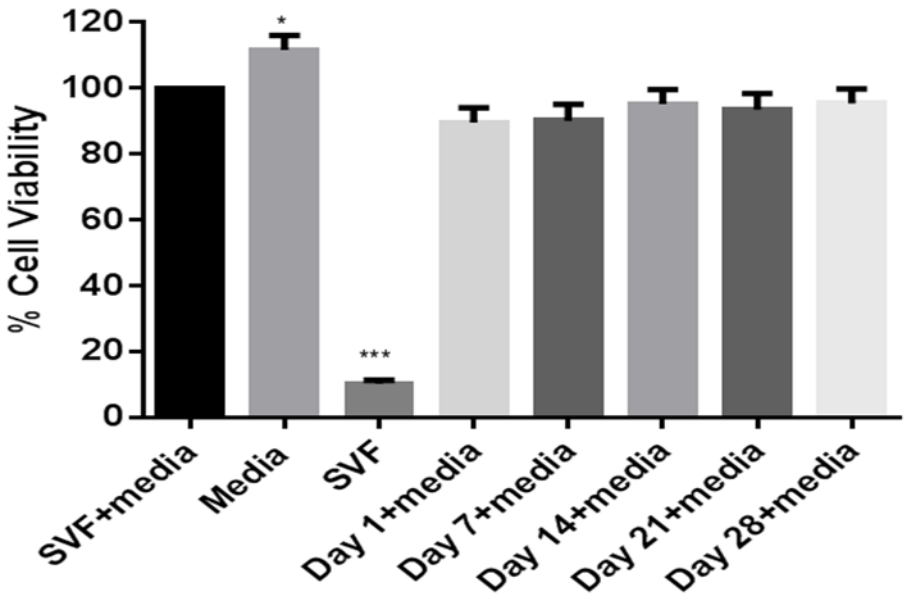

Figure 3: Viability of vaginal cell line VK2/E6E7 (ATCC® CRL 2616™) after exposure to simulated vaginal fluid (SVF) that had contained PCL matrices for 1 to 28 days (diluted 1:6 with culture media) (n = 3, mean ± s.d). SVF diluted with nutrient media (1:6), nutrient media alone and SVF were used as no treatment, positive and toxic controls, respectively. Data was compared to SVF + media (1:6) (*p<0.05, **p <0.01, ***p <0.001).

Lactobacilli are the most prevalent species in the vaginal environment and are considered important for maintaining the vaginal pH that acts as a major defensive mechanism against HIV and other pathogens[36]. It is important therefore that PCL matrices should not exhibit antimicrobial activity towards these commensal bacteria. No reduction in growth of *L. jensenii* was recorded when samples of PCL matrix were incubated in SVF for 28 days and the incubation media was tested against the lactobacilli in cell culture. An absence of significant levels of antimicrobial extractables is indicated, which provides further support for the favourable biocompatibility of PCL matrices and their intended application as vaginal delivery devices.

The shore hardness of 15% w/w doxycycline-loaded matrices increased by approximately 60% compared with drug-free matrices to around 1900 mN/mm^2 (Table 1), indicating that the drug particles in the PCL matrix act as reinforcing agents. However, the shore hardness is still more than 4 times lower than poly (ethylene vinyl acetate) IVRs

(Nuvaring®) which were characterised by a shore hardness value of 9280 mN/mm^2. Thus doxycycline-loaded PCL matrices may be expected to provide improved user comfort compared with conventional IVRs.

A burst release phase is evident over the first 24 h of testing when doxycycline-loaded PCL matrices were immersed in SVF at 37°C, resulting in a rapid loss of around 24-32% of the drug content (Figure 4A). Burst release is generally considered to result from dissolution of drug located at or close to the surface of the delivery device. Thus, the increase in burst effect with doxycycline loading of the matrices suggests a corresponding increase in surface presence of drug particles. Gradual drug release occurred over the following 13 days giving rise to an almost linear profile and total loss of 66-76% of the drug content. In PCL matrices with lower drug loading, drug particles are more separated within the microporous PCL matrix compared with the higher drug loaded samples, resulting in extended diffusion path lengths and lower concentration gradients that contribute to a reduction in drug release. In the case of higher drug loading, in addition to the increased concentration gradient for drug diffusion, the increased number of drug particles within the PCL matrix may give rise to micro-cracking effects. This behaviour has been suggested to form a system of interconnected pores and channels which form a pathway for the entry of release media, leading to an increase in the rate of drug dissolution and release[37].

The minimum amount of drug released on day 11 into 10 mL of SVF from 5% w/w doxycycline-loaded matrices was 0.3 mg (Figure 4B). This data provides an estimate of the drug dose which would be delivered from a PCL IVR device *in vivo* and the drug concentration that would be produced in vaginal fluid for assessment of the antibacterial effect.

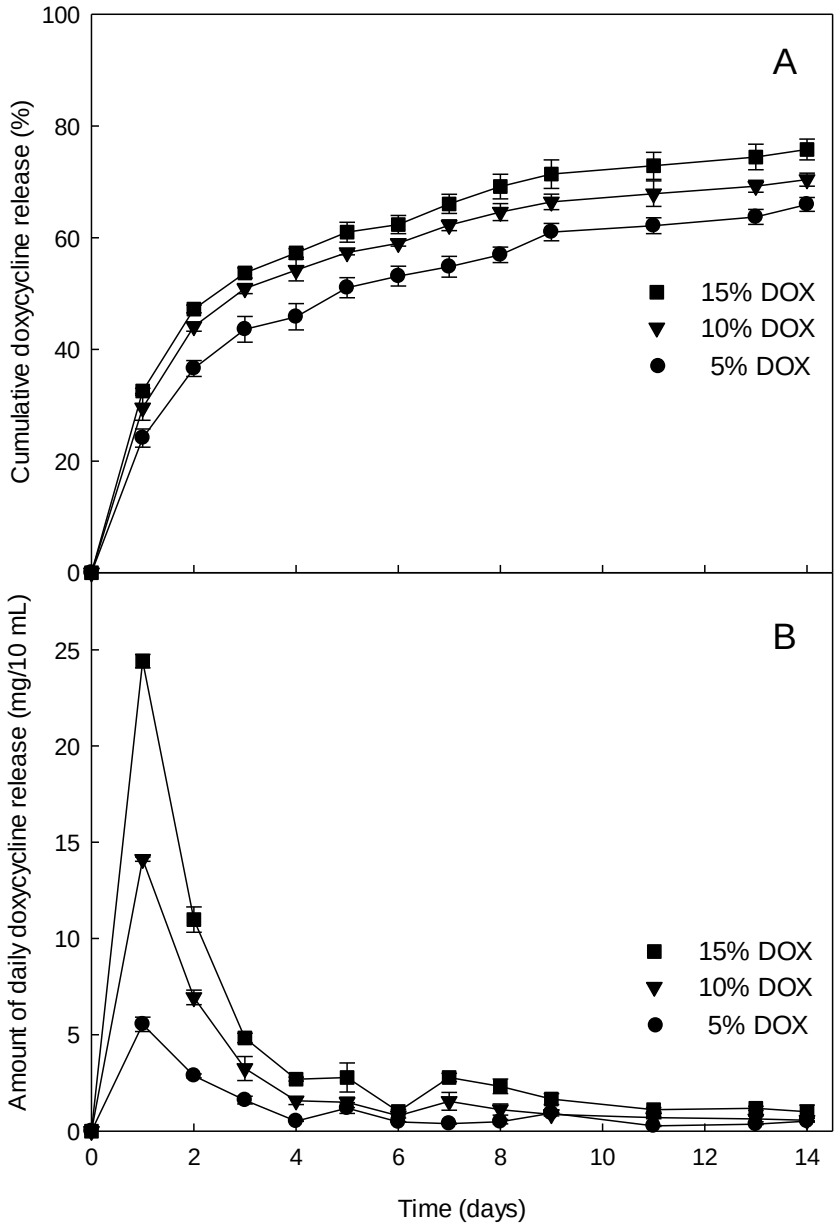

Figure 4: Cumulative (A) and amount (B) of doxycycline (DOX) release from PCL matrices into simulated vaginal fluid at 37°C

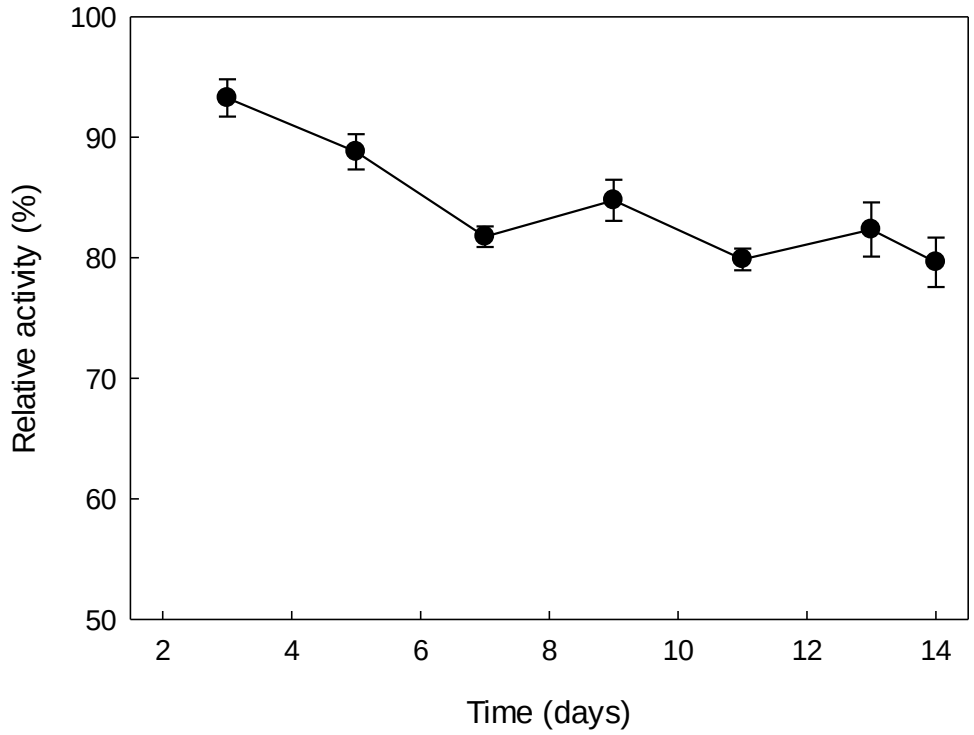

Figure 5: Antibacterial activity (%) of doxycycline released from 5% w/w doxycycline-loaded PCL matrices against *Neisseria gonorrhoea* relative to the standard non-formulated drug

The antibacterial activity of doxycycline solutions (non-formulated) increased linearly with drug concentration with R^2=0.9877 for the concentration range 30-150 µg/mL. The activity of doxycycline released from PCL matrices into SVF ranged from 90% on day 3 to 81% on day 14 (Figure 5) compared with non-formulated drug solutions of equivalent concentration. The small decrease in relative activity indicates that doxycycline is susceptible to degradation over time when exposed to SVF at 37°C within the matrix. The concentration of doxycycline in dry powder has been reported to reduce by 3% when stored at 40°C for 30 days[20]. The tetracyclines such as doxycycline hyclate are known to undergo reversible epimerisation at positions C-4 and C-6 under abnormal conditions of heat, pH and humidity to form a mixture of degradation products that have low antibiotic activity[38]. Thus the decrease in activity of doxycycline released from PCL matrices at extended time periods may be explained by gradual degradation associated with epimerisation.

Given that the minimum daily concentration of drug released into SVF by 5% w/w doxycycline-loaded matrices is 30 µg/mL (Figure 4B), the cylindrical test samples used in

this study (4.5 cm long) correspond to approximately one third of the linear length of an IVR, and the turnover rate of vaginal fluid is approximately 8 mL/day[27], the calculated minimum concentration of doxycycline released per day is 112.5 ug/mL. This far exceeds the minimum inhibitory concentration (MIC) against *N. gonorrhoea* (0.5-4.0 µg/mL)[39], *Chlamydia trachomatis* (0.016-0.064 µg/mL)[40] and *Mycoplasma genitalium* (0.125-0.25 µg/mL)[41]. This basic modelling approach neglects the changes in volume and biochemistry of vaginal fluid over time (for example, pH), which will influence drug release kinetics and local drug concentration, but it illustrates the potential of these materials for maintaining elevated levels of anti-bacterial concentration and activity over extended periods of time in vaginal fluid.

5 Conclusion

PCL matrices loaded with doxycycline are able to deliver the drug efficiently for up to 14 days in SVF with retained activity of 80-90% relative to (non-formulated) doxycycline solutions. The minimum concentration of released drug exceeded the MIC of doxycycline against many pathogens implicated in STIs. Biocompatibility studies of PCL further proved that the PCL is a safe polymer for the vaginal delivery as it has no effect on the vaginal cells and lactobacilli. These findings recommend further investigation of PCL matrices for vaginal delivery of antimicrobials as a strategy for local drug delivery, with the intention of improving patient compliance to reduce the risks of drug resistance and reinfection associated with long-term oral delivery. After studying the drugs individually further study was required to see the effect of combination of drugs on polymer and the release profile. In the next chapter, we tried to load combination of metronidazole and doxycycline.

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CHAPTER 4

SUSTAINED SIMULTANEOUS DELIVERY OF METRONIDAZOLE AND DOXYCYCLINE FROM POLYCAPROLACTONE MATRICES DESIGNED FOR INTRAVAGINAL TREATMENT OF PELVIC INFLAMMATORY DISEASE

1. Abstract

Poly(ε-caprolactone) (PCL) matrices loaded with a combination of antibacterials, doxycycline and metronidazole, were produced by rapidly cooling a mixture of drug powders dispersed in 15% w/v PCL solution in acetone. Matrices loaded with different combinations of metronidazole (10, 15 and 20%) w/w and doxycycline (10%) w/w, measured with respect to the PCL content, were evaluated for release behaviour and antibacterial activity. Rapid 'burst release' of 8-15% of the drug content for doxycycline and 31-37% for metronidazole was measured within 24 h when matrices were subjected to *in vitro* release testing in simulated vaginal fluid (SVF) at 37°C. The remaining drug was extracted gradually over 14 days to a maximum of 65-73% for doxycycline and 62-71% for metronidazole. High levels of antibacterial activity of up to 82-91% against *Gardnerella vaginalis* and 82-92% against *Neisseria gonorrhoeae*, were recorded *in vitro* for release media collected on day 14, compared to non-formulated metronidazole and doxycycline. The levels of both metronidazole and doxycycline released from PCL matrices into vaginal fluid *in vivo* were predicted to be greater by a factor of 2.5 and 7 than the minimum inhibitory concentrations for *N. gonorrhea* (0.5-4.0 μg/mL) and *G. vaginalis* (2-12.8 μg/mL) respectively, which are two of the major causative agents for pelvic inflammatory disease. These findings recommend in *vivo* investigations of PCL matrices loaded with a combination of doxycycline and metronidazole, in the form of intravaginal rings, for the treatment of pelvic inflammatory disease.

2. Introduction

Pelvic inflammatory disease (PID) is caused by migration of pathogenic and commensal microorganisms from the vagina or cervix towards the upper genital tract[1,2]. *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and many anaerobic bacteria may be found in the cervix, endometrium and fallopian tube of women suffering from PID and are considered to be the major causative agents[3]. Many non-gonoccocal, non-chlamydial microorganisms such as *Prevotella* species, Gram-negative anaerobic rods, *Peptostreptococcus* sp., *Gardnerella vaginalis, Escherichia coli* and aerobic streptococci are recognized as contributing to bacterial vaginosis and, in the presence of cervical infection/inflammation, may ascend to the upper genital tract and become implicated in PID[4]. Signs of PID include inflammation of the upper genital tract, endometritis, salpingitis, and pelvic peritonitis and if not treated can cause tubo-ovarian abscess, tubal factor infertility, ectopic pregnancy and chronic pelvic pain[5,6].

The most widely accepted treatment for PID involves oral doxycycline (100 mg) and metronidazole (400 mg) every 12 hours for a minimum of 2 weeks, since the combination displays activity against most of the bacteria that are the main causative agents for PID[7, 8]. However, patient non-compliance is common due to the long duration of treatment and the gastrointestinal side effects, oesophageal ulcers and bad taste associated with oral delivery of these drugs[9], which may lead to repeated episodes of PID and consequently a higher risk of infertility and other complications[10]. The rate of infertility has been reported to increase from 8-12% for one PID episode to 40-50% for three PID episodes[8]. The risk is accentuated for young people due to a cervicovaginal environment that is more sensitive to infection, engagement in high-risk sexual behaviour[6] and they are particularly non-adherent to the therapy[11]. There is, therefore, an impetus to treat PID efficiently by developing a drug delivery system that increases patient acceptance and thus compliance.

The vaginal route of drug administration has proven successful for contraceptive purposes (spermicidal and hormonal agents), potential prevention of HIV (tenofovir) and cancers of the reproductive tract (local delivery of cisplatin)[12-16]. Vaginal delivery offers widely recognized advantages over competing routes of administration including local drug delivery at the site of action in the case of HIV and other STIs, sustained drug delivery from controlled release devices, and the avoidance of gastrointestinal absorption and hepatic first pass effects leading to lower dosing and reduced side effects[17]. Vaginal delivery systems are available in the form of creams, gels, pessaries, films, tablets and intravaginal rings (IVRs). The advantages of IVRs over other approaches include the opportunity for sustained drug delivery and for avoiding the inconvenience and ‘messiness’ associated with insertion of semi-solid formulations (creams and gels)[13]. A year-long study carried out in 52 countries to check the efficacy, tolerability and acceptability of the Nuvaring® IVR revealed that out of the 806 women who completed the study, 96% expressed satisfaction with the ring performance and 98% would recommend the device to others[18]. In a randomized 12 week trial of the acceptability of silicone elastomer IVRs (without drug), involving HIV-negative women aged 18-35, of the 157 women who completed the trial, 96% reported finding the IVR acceptable for daily use[19]. Recent advances in IVR technology have demonstrated their potential utility for delivery of microbicides that can prevent the transmission of HIV/AIDS[20,21], but the use of IVRs for the treatment of curable STIs has received comparatively little attention.

The materials conventionally used for IVR production such as silicone elastomer and polyethylene vinyl acetate (pEVA) have the disadvantages of high processing

temperatures and hydrophobicity[22]. Silicone elastomer requires a curing reaction at 80°C, while pEVA is injection moulded at 140°C. Conversion of etonogestrel from the amorphous to the crystalline form at elevated processing temperatures has been reported on the surface of EVA copolymer at high drug loading[23]. Poly (ε-caprolactone) (PCL) offers advantages for the production of intravaginal drug delivery devices because of its biocompatibility, ease of processing and relatively low processing temperatures[24]. PCL has already been widely studied for controlled drug delivery in the form of micro- and nanoparticles, fibers and matrices[25]. In particular, PCL matrices prepared by rapid cooling techniques have been investigated for controlled vaginal delivery of individually loaded antibacterials (gentamicin and norfloxacin)[26,27] antifungal agents (miconazole)[27] and antiviral agents (tenofovir and acyclovir)[28,29]. Here we describe sustained, simultaneous delivery of metronidazole and doxycycline hyclate from PCL matrices containing both drugs. The materials are intended for production of IVRs in the treatment of PID, where doxycycline and metronidazole are active against most of the bacteria that are the main causative agents.

3. Materials and methods

3.1 Materials

PCL (Mw 115,000 Da, CAPA 6500) was obtained from Solvay Interox, Warrington, UK. Doxycycline hyclate was purchased from Alfa Aesar (Ward Hill, MA, USA) and metronidazole, sodium chloride, potassium hydroxide, calcium chloride, bovine serum albumin, glucose, glycerol, urea, lactic acid and acetic acid were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Solvents (acetone, acetonitrile) were of analytical grade. *Gardnerella vaginalis* stock culture in glycerol broth was supplied by Micromon, Monash University, Clayton, VIC, Australia. Horse blood agar, heart infusion broth (HIB), and CO_2 generating kit sachets were obtained from BD (New Jersey, USA), and Thayer Martin Agar and MRS broth from Micromedia, VIC, Australia. *Neisseria gonorrhoeae* NG1291 was provided by the School of Chemistry and Molecular Bioscience, the University of Queensland (UQ), Australia. *Lactobacillus jensenii* ATCC 25258, a human vaginal isolate, was sourced from the School of Agriculture and Food Sciences, UQ. The human vaginal epithelial cell line Vk2/E6E7 (ATCC® CRL2616™), Dulbecco's modified Eagle's media / F12 Media and DMSO were purchased from American Type Culture Collection (Manassas, Virginia, USA). Keratinocyte, serum-free

media (GIBCO^{TM}), human recombinant EGF, bovine pituitary extract, penicillin and streptomycin (P/S) were purchased from Sigma-Aldrich, Australia.

3.2 Production of PCL matrices loaded with metronidazole and doxycycline hyclate in combination

Cylindrical PCL matrices loaded with combinations of metronidazole and doxycycline hyclate were prepared according to our previously reported method (Chapter 2). A 15% w/v solution of PCL in acetone was produced by heating at 45°C for 45 min. Finely ground doxycycline and metronidazole powder were added to attain a drug loading of 10, 15 and 20% w/w metronidazole and 10% w/w doxycycline, measured with respect to the weight of PCL. A large burst effect has been observed for PCL matrices loaded only with metronidazole (35-65%) along with low drug release rates over a period of 14 days (Chapter 2), while in contrast doxycycline release from single-loaded PCL matrices was more consistent (Chapter 4), resulting in gradual delivery of 65-75% of the drug load over 14 days. Thus PCL matrices were loaded with increasing concentrations of metronidazole at constant doxycycline loading in the present study to focus on enhancing the rate and quantity of metronidazole release. The resulting drug suspension in PCL solution was poured into 3 mL polypropylene syringes as a mould and cooled rapidly at -80°C to induce crystallisation of the PCL phase. After retention at -80°C for 24 h the resulting PCL matrices were removed from the moulds and dried for 24 h under ambient conditions to evaporate solvents. The resulting cylindrical matrices had a diameter 6.3 ± 0.5 mm and length 45 ± 3 mm.

3.3 Morphology of drug-free and drug-loaded PCL matrices

Scanning electron microscopy (SEM) (JEOL-6610LV SEM, Jeol Ltd., Tokyo, Japan) was performed on drug-free and drug-loaded PCL matrices to examine the morphology of the matrices and the drug distribution within the matrix. Thin sections were mounted onto aluminum stubs using adhesive carbon tabs and sputter coated with platinum using an Eiko sputter coater prior to examination in the SEM at 10 kV.

3.4 Thermal properties of drug-free and drug-loaded PCL matrices

Differential scanning calorimetry (DSC) (DSC 1 STARe System, Mettler-Toledo Ltd., Victoria, Australia) was used to study the thermal properties of drug-free and drug-loaded PCL matrices. Samples (approximately 5 mg, accurately weighed) were sealed in aluminium pans and heated over the temperature range 30-330°C at a rate of 10°C/min.

The glass transition temperature (Tg), and crystallinity data were obtained using the DSC software facility. The percentage crystallinity of the PCL phase was estimated using a heat of fusion of 139.5 J/g for the fully crystalline polymer [26].

3.5 *In vitro* release behaviour of metronidazole and doxycycline from combination-loaded PCL matrices

Triplicate samples of drug-free and drug-loaded cylindrical matrices (6.3 mm diameter, and 45 mm length) containing metronidazole and doxycycline in combination were weighed and the ends were sealed using 5% w/v solution of PCL in acetone. Individual samples were placed in 10 mL of SVF and incubated at 37°C for 14 days. Release media were collected daily and replaced with fresh media. The concentration of metronidazole and doxycycline in the release media was measured using a Shimadzu HPLC equipped with binary pump and SPD-M20A photodiode array detector, and 2.1 X 150 mm, 5 µm Agilent Extend C-18 (Agilent Technologies, CA, USA). Gradient elution was performed using water (eluent A) and acetonitrile (ACN, eluent B) with 15% ACN concentration, increasing to 70% at 0.5 mL/min over 10 min, then held for 5 min. Detection was performed at 276 nm for doxycycline and 320 nm for metronidazole. Standard curves were generated for each drug using calibration samples ranging from 10-100 µg/mL and the concentration of each drug in the release media was obtained by comparison with the standard curve. Separate samples of release media were stored at 5°C prior to assay of antibacterial activity.

3.6 Antibacterial activity of metronidazole and doxycycline released from PCL matrices

The antibacterial activity of SVF release media collected over 14 days for PCL matrices loaded with 10% metronidazole and 10% doxycycline was assayed against *G. vaginalis* and *N. gonorrohoeae* using the disc diffusion method. The antibacterial activity of metronidazole and doxycycline in the release media was compared with the same concentration of non-formulated metronidazole or doxycycline dissolved in SVF. *G. vaginalis* was grown for 48 h at 37°C under anaerobic condition on horse blood agar plates while *N. gonorrohoeae* was grown on Thayer Martin agar plates for 24 h at 37°C under 10% CO_2. Colonies of both microorganisms were collected in HIB by scraping from the agar surface. The suspensions of *G. vaginalis* were subsequently diluted in HIB such that 100 µL plated on horse blood agar resulted in 100 colony forming units (CFU); the colonies for this strain were large and spreading and thus only a low inoculum was needed. The cell

suspension of *N. gonorrohoeae* was diluted to obtain an optical density of 1 at 550 nm and 100 µL of the suspension was spread on Thayer Martin agar. The disc diffusion assay was performed by placing a blank disc (Oxoid) at the center of each inoculated plate and adding 100 µL of SVF release media or non-formulated drug solution (standard) of the same concentration. Plates were incubated for 24 h and the diameter of the zone of inhibition surrounding each disc was measured. The relative antibacterial activity (%) of metronidazole and doxycycline released from the PCL matrices was obtained by comparing the zone of inhibition with that of non-formulated drug samples of equivalent concentration. SVF alone and SVF release media that had contained blank PCL matrix were used as controls.

4. Results and Discussion

4.1 Morphology of PCL matrices

PCL matrices containing doxycycline and metronidazole in combination were produced successfully by rapidly cooling a suspension of both drugs in PCL solution, and presented as uniform cylindrical mouldings with an absence of cracks and voids in the interior and on the surface. SEM examination revealed large numbers of drug crystals of size around 5 µm on the surface of PCL matrices (Figure 1A). Although it is not possible to distinguish between metronidazole and doxycycline crystals in the micrograph, the greater burst release of metronidazole (Section 4.5) suggests that the crystals are likely to be metronidazole. The size and morphology of metronidazole (100 µm) and doxycycline (10 µm) particulates used in matrix production are revealed in Figure 1D and 1E, respectively, indicating that the drug crystals are broken down during matrix formation production. The highly porous morphology of the PCL matrices revealed (Figure 1B) may be expected to facilitate gradual release of both antibacterials. The pore sizes ranged between 2-4 µm and a similar morphology is visible in the drug free PCL matrix (Figure 1C).

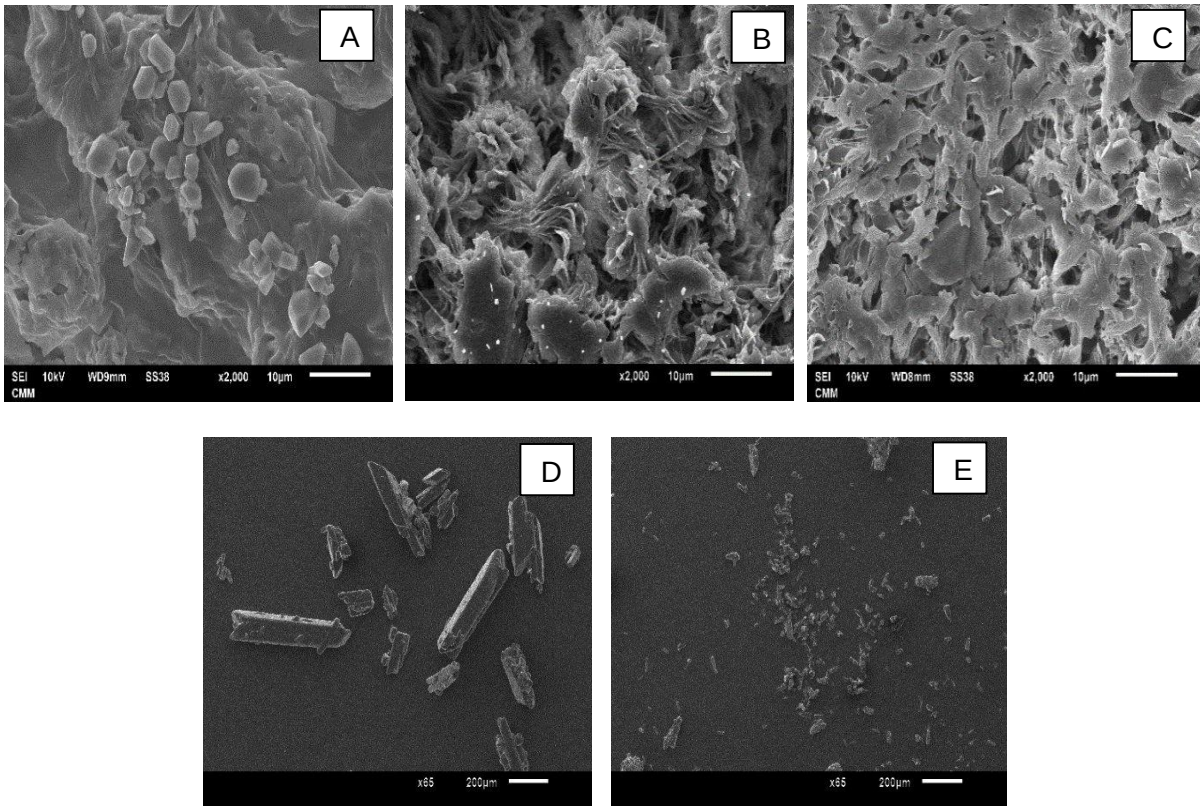

Figure 1: Scanning electron micrographs of PCL matrices A) Surface and B) Interior of PCL matrices loaded with 10% metronidazole, 10% doxycycline loaded PCL matrix C) Surface of blank PCL matrix D) Metronidazole crystals E) Doxycycline crystals

4.2 Thermal analysis

Thermal analysis of PCL matrices loaded with a combination of metronidazole and doxycycline revealed a small increase in glass transition temperature (Tg) of the PCL phase at the highest drug loading (Table 1) which may be explained by the reinforcing effect of the particulates and restriction of polymer chain mobility. The crystallinity of the PCL phase of drug-loaded matrices is expected to influence release characteristics and thus therapeutic efficacy; fluid transport is impeded by a close packed crystal structure, resulting in changes in drug dissolution and diffusion kinetics[31]. Crystallinity of the PCL phase was reduced by over 50% on incorporation of both metronidazole and doxycycline at loadings of 10% w/w (Table 1), indicating interference with PCL crystal nucleation and growth. When loaded individually into PCL, as loading increases, doxycycline reduces crystallinity (Chapter 3) while metronidazole increases crystallinity (Chapter 2) in comparison to drug-free PCL matrices. Indeed, in the present study PCL crystallinity increased as the concentration of metronidazole loaded into the matrices increased from

10 to 20% (Table 1), supporting the hypothesis that metronidazole acts as a nucleating agent for crystallisation of PCL. The increase in PCL crystallinity in matrices incorporating both metronidazole and doxycycline is less than with metronidazole alone (Chapter 2), providing further evidence that doxycycline is opposing the effect of metronidazole by restricting crystallisation of PCL (Chapter 3).

Table 1: Thermal analysis of PCL matrices loaded with a combination of 10, 15 and 20% w/w metronidazole (MTZ) and 10% w/w doxycycline (DOXY). The numbers sharing the same superscript letter are not significantly different (P<0.05).

Drug loading (w/w)	Glass transition temperature (°C)	Crystallinity (%)
Drug-free	-64.1 ± 1.3[a]	82.3 ± 4.3[a]
10% DOXY + 10% MTZ	-63.8 ± 2.1[a]	30.6 ± 0.9[b]
10% DOXY + 15% MTZ	-64.1 ± 0.8[a]	40.6 ± 2.4[c]
10% DOXY + 20% MTZ	-66.4 ± 1.1[a]	52.8 ± 1.3[d]

4.3 Release behaviour of doxycycline and metronidazole from PCL matrices loaded with a combination of both drugs

Cumulative release of metronidazole from PCL matrices loaded with a combination of metronidazole and doxycycline was characterized by a significant burst effect resulting in rapid loss of around 30-35% of the metronidazole content during the first 24 h in SVF (Figure 3A).

Gradual drug release occurred over the following 13 days resulting in delivery of 60-70% of the drug content. The amount of metronidazole released increased as drug loading in the matrix increased, indicating a diffusion-dominated release mechanism controlled by the drug concentration gradient. In the case of doxycycline, 8-15% release occurred in the first 24 h, which was smaller than that recorded in PCL matrices loaded with doxycycline alone (Chapter 4) and may indicate that the drug particles observed on the surface of the PCL matrices (Figure 2A) were predominantly metronidazole. Drug release occurred gradually until day 8 and then slowed, resulting in delivery of 65-75% of the doxycycline content after 14 days in SVF (Figure 2B). Doxycycline release increased with increasing loading of metronidazole, which may be explained by the formation of increasing numbers

of pores and channels as the drug loading in the matrix increased, facilitating ingress of SVF, and dissolution of drug particles and transport of drug molecules from the matrix.

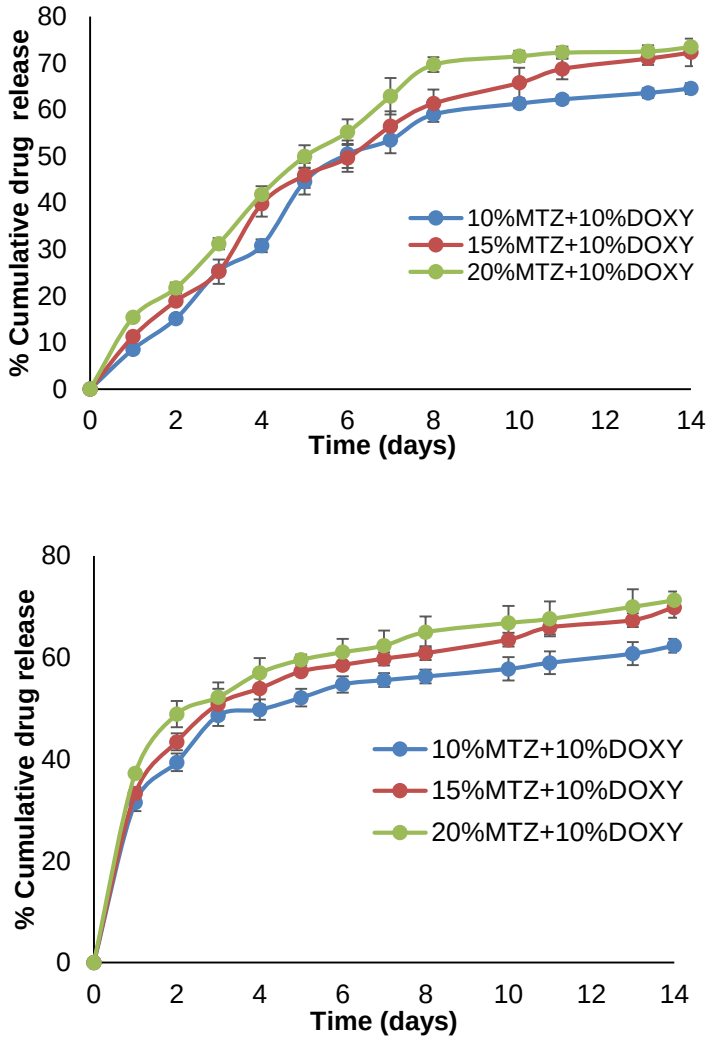

Figure 2: Cumulative release (% of total) into simulated vaginal fluid at 37°C of A) Doxycycline B) Metronidazole from PCL matrices loaded with a combination of metronidazole (MTZ) and doxycycline (DOXY)

The absolute amount of metronidazole and doxycycline released over time from PCL matrices loaded with a combination of both drugs is presented in Figure 3A and B respectively, which is useful for estimating the delivered dose from PCL matrices and for predicting the *in vivo* concentration of antibacterials in vaginal fluid. Approximately 15 mg of metronidazole was released into 10 mL of SVF at day 1 from matrices loaded with 10% w/w metronidazole and 10% w/w doxycycline and the amount fell to around 1 mg at day 14. Doxycycline release was lower in day 1 (5 mg) reflecting the lower burst effect but

reached a similar level to metronidazole at day 14. An erratic 'cyclical' release behaviour of doxycycline (Figure 3A) may reflect the lower solubility of doxycycline in SVF compared with metronidazole. The process of doxycycline release may be controlled by dissolution and release of metronidazole that subsequently permits ingress of SVF and dissolution of doxycycline particles. Overall, it is apparent that PCL matrices loaded with a combination of doxycycline and metronidazole are able to deliver both drugs gradually into SVF for 14 days with a relatively high delivery efficiency of 70%.

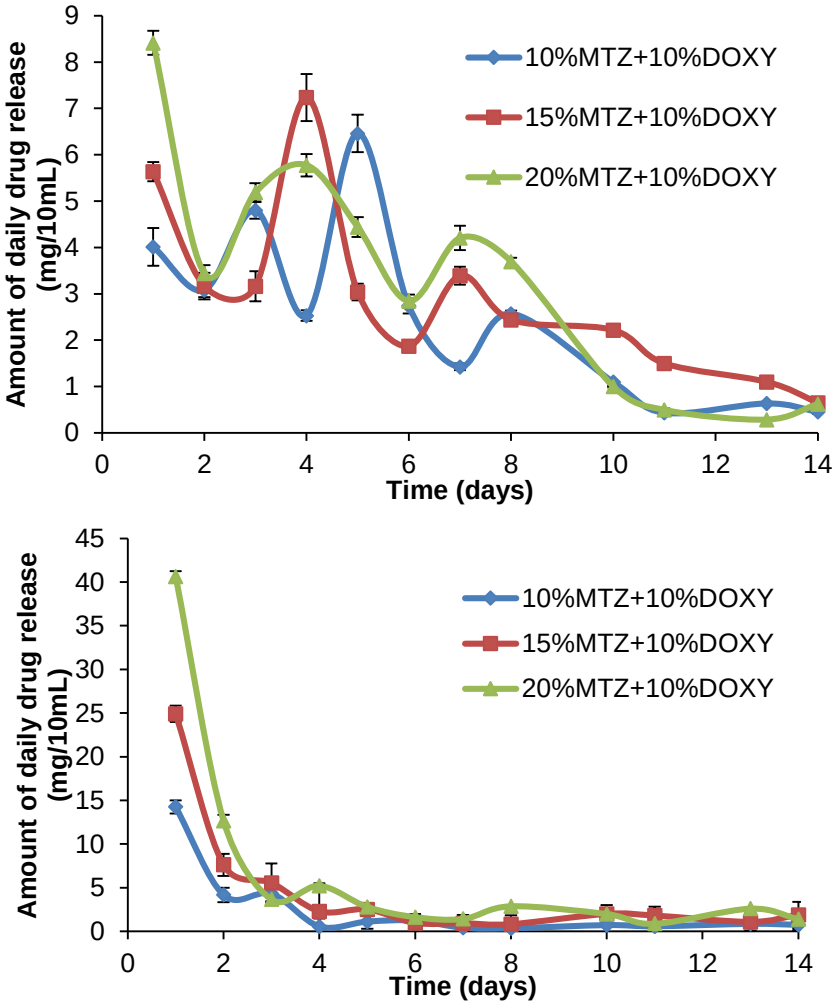

Figure 3: Amount (mg) A) of Doxycycline (DOXY) and B) Metronidazole (MTZ) released daily from PCL matrices into the 10 mL simulated vaginal fluid at 37°C

4.4 Antibacterial activity of metronidazole and doxycycline released from PCL matrices against *G. vaginalis* and *N. gonorrhoeae*

The antibacterial activity of metronidazole and doxycycline released on day 14 from PCL matrices loaded with a combination of both compounds was assessed to investigate the effect of matrix production and residence time in SVF on antibacterial activity. Zones of inhibition for *G. vaginalis* cultures on agar increased linearly from 7 to 20 mm when increasing concentrations of non-formulated metronidazole in SVF (25-125 µg/mL) were applied. Similarly, the zone of inhibition for *N. gonorrhoeae* cultures on agar increased linearly from 17-28 mm when increasing concentration solutions of non-formulated doxycycline in SVF (20-100 µg/mL) were applied. No zone of inhibition was observed for SVF alone or release media obtained from drug-free PCL matrices. High relative antibacterial activity was measured for SVF containing metronidazole and doxycycline released from PCL matrices loaded with both drugs at a 10% loading level; activity was 86-96% with respect to non-formulated metronidazole and 84-92% with respect to non-formulated doxycycline (Figure 4). The relatively small decrease in antibacterial activity over the course of the study may be due to prolonged exposure of the antibacterials within the PCL matrix to warm temperature (37°C) and SVF causing partial degradation. The tetracyclines such as doxycycline hyclate are known to undergo reversible epimerisation at positions C-4 and C-6 under abnormal conditions of heat, pH and humidity to form a mixture of degradation products that have low antibacterial activity (Chapter 3), and a 1% reduction in metronidazole content has been reported when subjected to accelerated stability conditions of pH 2-6 at a temperature of 25 and 37°C in aqueous conditions[32].

The minimum daily amount of metronidazole and doxycycline released from PCL matrices (length 45 mm) into 10 mL SVF was measured as 30 µg metronidazole at day 11 from 15%MTZ+10%DOXY w/w matrices and 27 µg doxycycline at day 13 from 20%MTZ+10%DOXY w/w matrices (Figure 3A and B). Based on this, the estimated drug concentrations that would be generated in vaginal fluid *in vivo* using an IVR produced from a PCL matrix, is anticipated to far exceed the minimum inhibitory concentration (MIC) for metronidazole against *G. vaginalis* (2–12.8 µg/mL)[33]. Similarly, the MIC for doxycycline against *N. gonorrhoea* (0.5-4.0 µg/mL)[34], *Chlamydia trachomatis* (0.016-0.064 µg/mL)[35] and *Mycoplasma genitalium* (0.125-0.25 µg/mL)[36] would be exceeded. These predictions are based on the linear length of an IVR of 150 mm and a vaginal fluid turnover rate of 8 mL per day[37] and neglects the complex changes in vaginal fluid over time (for example, pH, biochemistry and volume) that may influence drug release behaviour. However, the

demonstrated drug release and activity profiles *in vitro* underline the potential of PCL matrices for sustained intravaginal delivery of a combination of antibacterials for the treatment of PID.

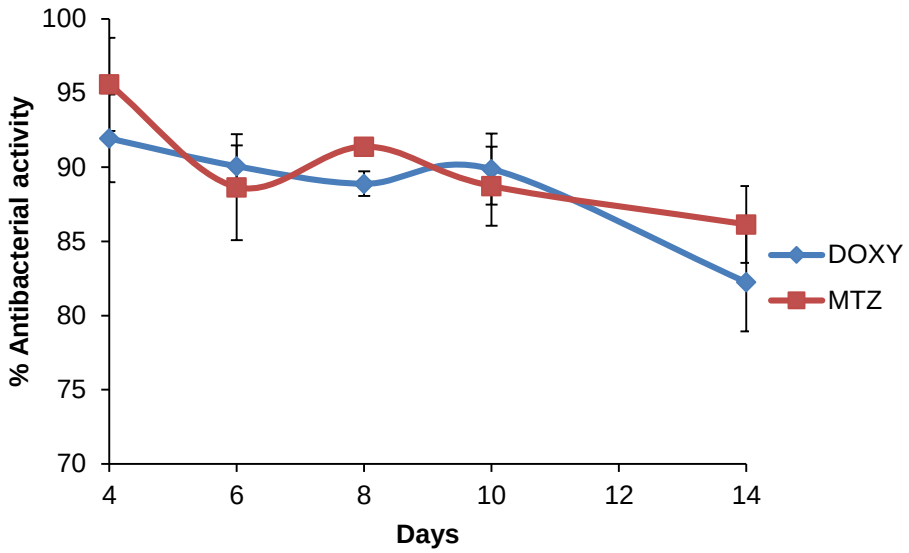

Figure 4: Relative antibacterial activity of doxycycline (DOXY) and metronidazole (MTZ) released from PCL matrices incorporating both antibacterials compared with the non-formulated drugs

5. Conclusion

PCL matrices loaded with metronidazole and doxycycline in combination provide simultaneous and sustained release of each antibacterial for up to 14 days in simulated vaginal fluid with antibacterial activity against *G. vaginalis* and *N. gonorrohoeae* in excess of 80% of the non-formulated drugs. The concentration of metronidazole and doxycycline achieved *in vitro* exceeded the MIC required to eradicate many pathogens implicated in PID. Good drug loading obtained for the combination of two drugs further confirming the suitability of the improved production method. The above results again confirm the suitability of PCL polymers for the delivery of combination of antibacterials.

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CHAPTER 5

INVESTIGATION OF INTRAVAGINAL DELIVERY OF A MACROMOLECULE USING POLYCAPROLACTONE

1. Abstract

Polycaprolactone (PCL) matrices loaded with lactoferrin, as a model macromolecule, were prepared using a rapid cooling method. Matrices loaded with 5% and 10% w/w lactoferrin were studied for matrix properties and lactoferrin release. A comparatively fast release of around 13-14% occurred on day 1, with 90-95% protein being released within 14 days in simulated vaginal fluid (SVF) at 37°C. Using SDS-PAGE, it was determined that the lactoferrin released from PCL matrices into SVF was intact. Differential scanning calorimetry indicated a decrease of 6% in crystallinity and hardness testing showed that there was a 58% decrease in shore hardness for matrices loaded with 10% w/w in comparison to blank matrices, which suggests that lactoferrin causes some deterioration of polymer properties.

2. Introduction

Vaginal drug delivery systems have been used for contraception and for treatment and prevention of sexually transmitted infections. The vagina has potential as a drug delivery route for other medications because of its advantages over the other delivery routes such as avoidance of first pass metabolism, avoidance of GI side effects and less frequent and lower dosing[1]. The delivery of drugs used for the treatment of sexually transmitted infections through vaginal route also have the advantage of target delivery at the entry point of pathogens and direct contact of the drugs with the infected cells[2]. Many new drugs of biotechnological origin such as Enfuvirtide, 2F5 and recombinant proteins gp160, gp140 and gp41[3-5] for the treatment of sexually transmitted infections are macromolecular in nature and new strategies are being investigated toward the delivery of these macromolecular drugs related with the prevention and treatment of STIs in an efficient and safe way through the vaginal route[6].

Recently, it has been reported that monoclonal antibodies (mAbs) such as 2F5, 4E10 and 2G12 protected macaques from the simian/human immunodeficiency viral (SHIV) challenge on IV, oral, rectal and vaginal application[7-9]. These mABs were found to be active against a wide variety of HIV isolates so could be potential for the treatment of HIV[10]. 5P12-RANTES and 6P4-RANTES are two recombinant proteins isolated from human chemokine proteins PSC-RANTES, found to exhibit anti-HIV by blocking the HIV coreceptor CCR5[11]. Attempts have been made to delivery various therapeutically important drugs such as insulin, calcitonin and sex hormones via the vaginal route but

there has been little success in the development of safe and viable vaginal formulations for these macromolecular drugs.

The human vagina is considered to be an alternative route for the delivery of various macromolecules such as insulin, calcitonin and sex hormones[12] and it has been found that molecular weight cut-off for vaginal mucosa is much higher than the other mucosal routes[13]. Many attempts have been made to deliver macromolecules for the treatment of sexually transmitted infections. CN54gp140, which is a HIV-1 envelope glycoprotein, has been delivered as a lipososme gel formulation in order to develop vaccination against HIV-1. An encapsulation efficiency of 35% was reported and the protein maintained more than 80% of their antigenicity[14]. Niosomes containing insulin prepared using sorbitan monoester as a carrier and by lipid evaporation, had entrapment efficiency of 17-28% and 30% of the entrapped insulin was released within 25 h. When these niosomes were studied for their efficiency in controlling blood glucose level in mice it was found that maximal hypoglycemic effects (a decrease of 46%) were observed within 1.5 h and the effect was retained for 6 hrs[15].

Intravaginal rings (IVRs) are considered to be the most acceptable and efficient vaginal drug delivery system for the delivery of many drugs such as antifungals, contraceptives and drugs related with HIV prevention[1]. They are particularly more acceptable because of ease of insertion, long term sustained delivery and avoidance of messiness caused by other conventional vaginal preparations such as vaginal creams[16]. The concept of delivering macromolecular drugs from IVRs is not new; previously protein antigen BSA in the form of inserts has been delivered using silicone elastomer with 80% protein release over a 12 week period and good protein stability[17]. Similarly a 10-pod insert IVR for the delivery of antibody IgG sustained *in vitro* release of 0.5-30 mg/day for 14 days and the IgG retained activity during the release period according to an ELISA test[18].

Currently polymers that are used in the manufacturing of intravaginal rings are silicone and polyethylene vinyl acetate, but these are almost impermeable to macromolecular drugs and are non-biodegradable. Polycaprolactone (PCL) on the other hand is a highly porous material that has been investigated for the intravaginal delivery of small molecules such as metronidazole and/or doxycycline for the treatment of sexually transmitted infections (Chapters 2-4). PCL never studied for the intravaginal delivery of macromolecules. The aim of the current study was to investigate PCL as a potential polymer for the delivery of a macromolecule. In the current study lactoferrin was used as a model macromolecule.

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein of the transferrin family that is expressed in most biological fluids and is a major component of the mammalian innate immune system[19]. This glycoprotein is found in mucosal secretions, including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids, urine and most highly in milk and colostrum[20].

3. Materials and methods

3.1 Materials

Polycaprolactone (PCL, MW 115,000 Da, Capa 650) was obtained from Solvay Interox (Warrington, UK). Bovine lactoferrin was a gift from Dr. Nidhi Bansal (Lecturer, School of Agriculture and Food Science, University of Queensland). Sodium chloride, potassium hydroxide, calcium chloride, bovine serum albumin, glucose, glycerol, urea, lactic acid and acetic acid were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Solvents (acetone, acetonitrile) were of analytical grade and obtained from purchased from Sigma-Aldrich, Australia. 2X Laemmli sample buffer, 12% SDS precasted 15 well gels were purchased from Bio-Rad (Gladesville, NSW, Australia). PageRuler plus prestained protein ladder was purchased from ThermoFisher Scientific (Scoresby, Vic, Australia).

3.2 Preparation of lactoferrin-loaded PCL matrices

1.5 g of PCL melted in 10 mL acetone at 45°C for 45 min. Once melted the resulting suspension was taken away the heat source and allowed to cool to 40°C to prevent the lactoferrin from excess of heat, and lactoferrin powder was dispersed into polymer suspension to produce protein loading of 5% and 10% w/w with respect to PCL content. Solid matrices of the polymer were obtained using 3 mL polypropylene syringe barrels as moulds as previously described (Chapter 4 and 5). The final matrices were in the form of cylinders of diameter 6.3 ± 0.2 mm and length 45.0 ± 3.0 mm.

3.3 Morphology

JEOL-6610LV scanning electron microscopy (SEM) (Jeol Ltd., Tokyo, Japan) was used to study the morphology of lactoferrin loaded PCL samples. Small samples were cut from the surface and inside the PCL matrices and fixed onto aluminum stubs and then coated using an Eiko sputter coater automatic mounting press. Coated samples were then studied for their surface and interior morphological characteristics using a voltage of 10 kV.

3.4 Thermal analysis

Differential scanning calorimetry (DSC) (DSC 1 STARe System, Mettler Toledo, Mettler-Toledo Ltd., Victoria, Australia) was used to study the effect of lactoferrin loading on the PCL matrices. Small pieces of PCL matrices loaded with lactoferrin were cut, weighed, sealed inside alumina pans. Samples were heated across the temperature range 30-330°C at a rate of 10°C/min. The glass transition temperature (Tg), and crystallinity data were obtained using the DSC software.

3.5 Shore hardness

Shore hardness of the of drug-free and lactoferrin-loaded matrices was carried out using a CT3 Texture Analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA). Cylindrical PCL matrices were mounted horizontally and compressed locally at a speed of 0.1 mm/min to a depth of 2.0 mm using a 2 mm diameter, flat-ended, cylindrical probe (TA39). The shore hardness (or indentation resistance) of each sample was calculated from the applied force measured at a depth of 2 mm. A pEVA IVR (Nuvaring®, Schering-Plough Pty limited, New South Wales, Australia) was subjected to the same test procedure for comparison.

3.6 *In vitro* lactoferrin release

Release of lactoferrin from PCL matrices was tested in simulated vaginal fluid (SVF) as the release media. Ends of the matrices were sealed with 5% w/v PCL solution to mimic the ring characteristics and to avoid any edge release of lactoferrin. Samples were put into 10 mL of SVF in an incubator at 37°C for 14 days. Each day samples of SVF containing released lactoferrin were collected and replaced with fresh 10 mL SVF. The concentration of lactoferrin in collected media was analyzed by using Shimadzu HPLC equipped with binary pump and SPD-M20A photodiode array detector. Separation was carried out using an Agilent Extend C-18 column (2.1 x 150 mm, 5 µm). Two mobile phases were used, water (eluent A) and 95% acetonitrile (eluent B), both with 0.1% (v/v) trifluoroacetic acid added A gradient elution started at 15% B and reached 70% B at 10 min and held at this level for 5 min. The column was re-equilibrated at 15% B for 10 min between runs.

The sample injection volume was 50 µl and the quantification of lactoferrin was performed at 276 nm against a standard curve for the concentration range 10-60 µg/ml (R^2 = 0.9988). Each experiment was performed in triplicate. Release samples were stored at 0°C for antibacterial testing.

3.7 SDS-PAGE of released lactoferrin

Small discs of PCL matrices loaded with 5% and 10% w/w lactoferrin were cut and incubated with 10 ml of SVF at 37°C for 14 days and the media then collected and tested for the stability of protein. 5 µL of the release media containing lactoferrin was mixed with 5 µL of sample buffer (4.5 µL of 2x Laemmli Sample Buffer and 0.5 µL of beta mercaptoethanol). 5 µL of the mixture was added into each well of a 12% SDS-PAGE gel and electrophoresis was performed according to Laemmli (1970)[21].

3.8 Statistics

Significant difference in glass transition temperature, crystallinity and shore hardness were tested using one-way ANOVA with a Tukey multiple comparisons test ($p<0.05$).

4. Results and discussion

4.1 Morphology of the PCL matrices

PCL matrices produced by rapid cooling exhibited a porous morphology with many interconnected pores and channels. The pore size in the case of blank matrices was 3-5 µm (Figure 1a) but this increased to 6-10 µm when lactoferrin was loaded (Figure 1b). Lactoferrin crystals, which vary in size from 0.5-3 µm (Figure 1c), were present on the surface of the matrix.

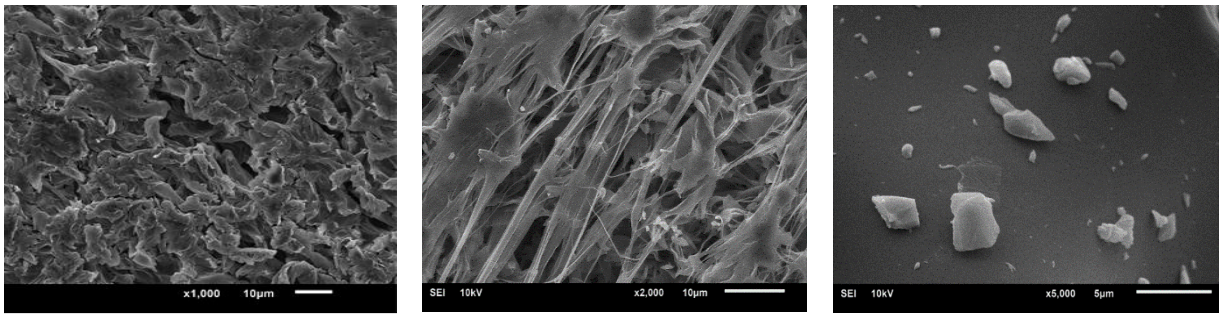

Figure 1: Surface view of a) Blank PCL b) PCL loaded with 5% w/w lactoferrin c) lactoferrin crystals

4.2 *In vitro* release characteristics

A fast release of lactoferrin was observed for the first 2 days for matrices loaded with 5% lactoferrin, and for the first 4 days for matrices containing 10% lactoferrin (Figure 2). Thereafter release occurred more slowly until 90-95% of lactoferrin had been released by the end of the 14 day test period. The more prolonged burst release exhibited by the 10%

lactoferrin-loaded matrices resulted in 70% release occurring by 4 days, while the slower release of the 5% lactoferrin-loaded matrices reached 70% at 7 days. The difference is due to more drug being available in the case of the higher loading. Initial faster release is a common feature of these types of polymeric systems and can be due to lactoferrin molecules coming to the surface of the polymer during the crystallization process. Gradual release of the protein is expected through the interconnected pores and channels in microporous PCL matrices and due to the microcracking effect on the internal structure of the polymers caused by protein particles[22].

Lactoferrin maintained its molecular size during matrix production and release into SVF. One protein band was observed for lactoferrin using SDS-PAGE electrophoresis, at a comparable location to lactoferrin standard (Figure 3), indicating that the structure of the protein that was released from PCL matrices into SVF over a 14 day period of time was not degraded into subunits. This assessment does not indicate the maintenance of activity, and this would be important to determine if a protein is to be loaded with an specific activity in mind; in this case lactoferrin was used as a model protein and this initial data indicates that the protein retained its structure. There is no previous research using lactoferrin with PCL, but using another model protein, bovine serum albumin, stability when released from PCL scaffolds formed by melt extrusion and PCL microparticles prepared by a w/o/w method was confirmed using circular dichroism[23,24].

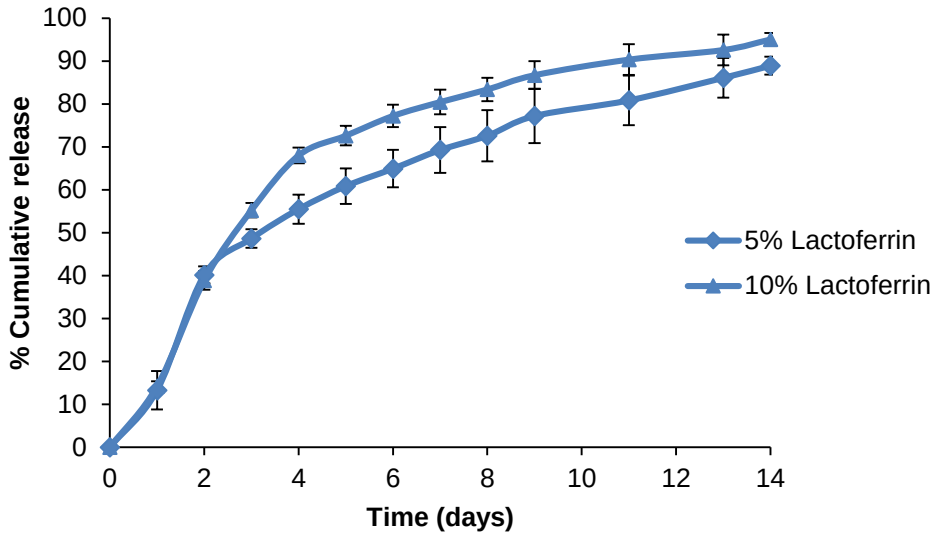

Figure 2: Cumulative release of lactoferrin (% of total loaded) from PCL matrices containing 5 or 10% w/w lactoferrin, measured in SVF at 37°C for 14 days.

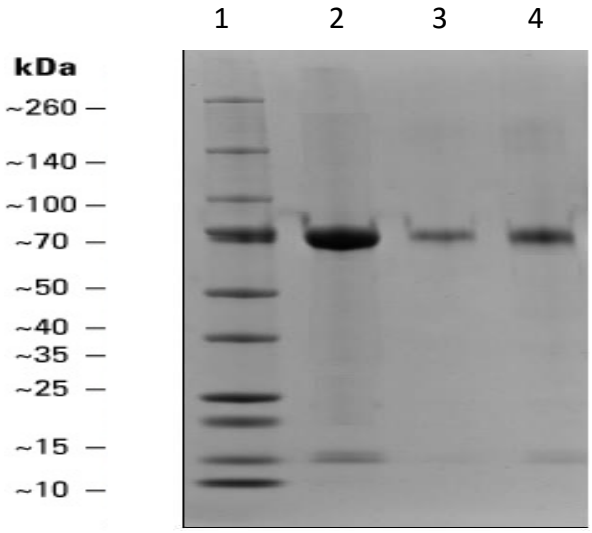

Figure 3: SDS-PAGE of lactoferrin (80 kDa): 1) Molecular weight biomarker ladder 2) lactoferrin standard 3) 5% w/w and 4) 10% w/w of lactoferrin after release from the PCL matrices into simulated vaginal fluid over 14 days.

4.3 Thermal characteristics and shore hardness

It is very important to study the effect of lactoferrin on the PCL polymer as it affects polymer degradation and hardness, and lactoferrin release. The main features that represent the thermal behaviour of the polymer are crystallinity and glass transition temperature. The effect of drug loading on the polymer can be studied by comparing the values with the blank polymer. A reduction in crystallinity of around 6% occurred when the

concentration of lactoferrin increased from 5 to 10% w/v (Table 1). The reduction in crystallinity indicates that protein molecules are interfering with crystal growth and nucleation of PCL.

Glass transition temperature (Tg) indicates the reversible change of the amorphous region of a polymer from viscous, to rubbery, and finally to a hard and brittle condition. There was a large (10°C) difference in Tg between blank matrices and lactoferrin-loaded matrices (Table 1), but minimal difference when the concentration of lactoferrin was increased from 5 to 10% w/v. This indicates that dispersing lactoferrin molecules into the PCL had an effect on PCL chain mobility. The crystallinity of the polymer decreased when the concentration of the polymer increased (Table 1) which might be due to suspended lactoferrin inhibiting PCL nucleation and crystal growth. The effect of added molecules on PCL matrix crystallinity varies with molecule loaded, as doxycycline caused a decrease in crystallinity of around 5% on increasing the concentration from 5% to 15% w/w (Chapter 3) but metronidazole loading was associated with an increase in crystallinity from 33% to 76% with increasing metronidazole loading of the material from 0 to 8.1% w/w (Chapter 2).

Matrix shore hardness decreased as lactoferrin loading increased (Table 1), representing the increasing amount of protein particles in the polymer having an increased reinforcing effect resulting in a softer matrix. However, the shore hardness of the material is far lower that one of the commercial vaginal rings available in the market i.e. Nuvaring®, for which shore hardness measured 9280 mN/mm^2 using the same method (Chapter 2). Using a soft material such as PCL will ensure the rings will be more comfortable to use then the conventional vaginal rings.

Table 1: Thermal and morphological analysis of PCL matrices loaded with lactoferrin. Within each column, numbers sharing the same superscript letter are not significantly different (P<0.05).

Protein loading (%w/w)	Glass transition Temperature (°C)	Crystallinity (%)	Shore Hardness (mN/mm^2)
0	-66.48 ± 1.2^a	82.32 ± 2.3^a	1382 ± 132^a
5	-55.23 ± 0.8^b	82.10 ± 4.6^a	1097 ± 87^b
10	-55.91 ± 0.9^b	76.72 ± 3.3^a	810 ± 62^c

5. Conclusion

PCL matrices are capable of loading lactoferrin without affecting its structural integrity. Extended release of lactoferrin over 14 days in SVF is possible. Lactoferrin is reported to have activity against many pathogens responsible for STIs[20, 25, 26] so could be a useful model for further investigation. This study provides avenues for further investigation into the use of this polymer in form of IVRs for delivering macromolecules for the treatment and prevention of sexually transmitted infections. In the current study we used SDS-PAGE to determine the structure of released lactoferrin but this technique does not provide any information about the secondary structure or activity of lactoferrin. Therefore investigation into the effect of PCL processing conditions on the structural integrity of lactoferrin molecules is highly recommended.

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CHAPTER 6
GENERAL DISCUSSION AND FUTURE DIRECTION

1. Introduction

The aim of this thesis was to investigate the potential for using polycaprolactone (PCL) as a matrix for controlled drug delivery for the intra-vaginal treatment of curable sexually transmitted infections. Metronidazole and doxycycline, separately and combined, were used because vaginal delivery of these agents would be beneficial in the treatment of bacterial vaginosis, gonorrhea and pelvic inflammatory diseases. The use of PCL for the delivery of a model macromolecule (lactoferrin) was investigated because macromolecular drugs, such as monoclonal antibodies and recombinant proteins, are in development for the treatment and prevention of STIs. There are a variety of intravaginal formulations available in the market for the treatment of STIs, but these are gels, creams, pessaries that require daily insertion. There are a small number of IVRs available commercially but these are for delivery of hormones for contraception and treatment of menopausal symptoms. This thesis draws attention to the potential use of PCL in intravaginal delivery of agents for the treatment of STIs.

2. PCL matrices for the delivery of different types of drugs

Many polymers have been tested for their potential use for intravaginal delivery but they all have some limitations, for example most of them can deliver only hydrophobic drugs, have a high processing temperature and are almost impermeable to macromolecular drugs. PCL on the other hand can deliver a wide variety of drugs molecules, including hydrophilic, hydrophobic and macromolecular structures, and has a low processing temperature which is advantageous for thermolabile drugs. This thesis firstly focused on metronidazole, a broad spectrum antibacterial having antimicrobial activity against most Gram-negative and Gram-positive anaerobic bacteria and used for the treatment of vaginal infections such bacterial vaginosis and trichomoniasis[1]. The standard oral treatment involves the use of metronidazole with an initial induction therapy for 7 days but a long-term maintenance regimen of metronidazole is advised. Oral administration of metronidazole is often associated with gastrointestinal adverse effects such as nausea, anorexia and occasionally vomiting and occasionally a bitter or metallic taste. Gastrointestinal side effects are reported to be less common with vaginal administration using the gels, creams and pessaries that are currently available, so long term vaginal delivery for the treatment of bacterial vaginosis could be very useful. Doxycycline is recommended for the treatment of chlamydial and gonococcal infection[2,3], with gastrointestinal side effects such as nausea, vomiting and mild diarrhoea being common, but it has not previously been

investigated for vaginal delivery. Metronidazole and doxycycline in combination are used for the treatment of pelvic inflammatory disease[4], with treatment being a minimum of 2 weeks in length.

In addition to studying the effect of processing and solvents on polymer characteristics, their effect on drug activity must be considered, so those aspects were included in this thesis. The amount of metronidazole and doxycycline released each day, both alone (Chapter 2 and 3) and in combination (Chapter 4), was more than the minimum inhibitory concentration against the main causative agents of bacterial vaginosis, gonorrohea and pelvic inflammatory diseases. Furthermore, the drugs retained high antibacterial activity after being loaded into and released from the polymer. It should be noted that these estimates were based on a single SVF pH value, and on the fact that the SVF turnover in the vagina of 'normal' women is said to be 8-10 mL/day, however other factors such as change in vaginal environment during menstruation, ageing and diseased conditions were not considered in this thesis. Therefore, it is recommended that before drawing any final conclusion further investigation is required to determine the extent to which these factors can alter the formulation, dosage and release profile.

In Chapter 2 the low incorporation efficiency of metronidazole was due to it dissolving and being removed in the ethanol used during the manufacturing process, and PEG only partially improved this, so during the study of doxycycline the step causing drug loss was omitted. This achieved 100% incorporation efficiency without losing any porous properties of the polymer (Chapter 3). A combination of two different drugs for treatment purposes could be an alternative that can enhance the spectrum of activity. Combinations of different drugs with different modes of action through vaginal rings have been applied previously in the study of HIV prevention, i.e. dapivirine with darunavir in a silicone IVR[5] and tenofovir with nevirapine in PCL matrices[6]. This thesis describes the first investigation of a combination of drugs for intravaginal drug delivery in the form of IVRs for the treatment of sexually transmitted infections, combining metronidazole with doxycycline for the treatment of pelvic inflammatory disease (Chapter 4).

Macromolecular drugs of biotechnological origin have been studied for the treatment and prevention of STIs such as Enfuvirtide, 2F5 and recombinant proteins gp160, gp140 and gp41[7-9], and an efficient polymeric delivery system is needed that can deliver macromolecules such as these. So experiments to investigate the efficiency of PCL for the delivery of a macromolecule are described in this thesis (Chapter 5). Lactoferrin was

used as the model drug because of its macromolecular nature and its reported activity against many infections. Lactoferrin was successfully loaded, and the ability to ensure that it did not exceed 37°C during processing was an important aspect of the potential value of PCL in delivery. However, the solvent used during processing, acetone, may not be appropriate for proteins as it may alter their structure, and although the molecular size of lactoferrin was unchanged further work is required to determine whether the secondary structure and/or activity is affected.

3. PCL as a potential material for the IVRs

A particularly important aspect of any matrix that is going to be used in or on a human being is biocompatibility with the appropriate tissues. Leachates from blank PCL matrices were tested for biocompatibility in terms of toxicity to vaginal cells and a key member of the normal vaginal flora. Data in this thesis (Chapter 3) provides evidence that these polymers are expected to be safe for vaginal drug delivery with no toxic effect on the vaginal cell line or *Lactobacillus jensenii*.

It is well established that IVRs are widely preferred as a vaginal preparation when compared to creams[10], a diaphragm[11] and a vaginal patch[12]. Women generally report being satisfied with IVRs and would continue to use them or recommend them to others[13,14]. The main concern about the use of IVRs tends to be some discomfort experienced by some women due to presence of the ring inside the vagina. The use of a soft polymer is also important to avoid pressure-induced vaginal lesions that can increase the chance of infection. This can be minimized by careful selection of the polymer, by choosing a polymer that is soft but still has the efficiency to deliver drugs over a long period of time. So in this thesis PCL was found to be a potential polymer because during the texture analysis (Chapters 2-5) PCL was found to be a softer material when compared with the Nuvaring®, which is commercially available polyethylene acetate IVR.

While PCL appears to be soft enough to be a suitable material for IVRs, all testing in this thesis involved cylinders of PCL matrix. Further investigation is required to determine whether it is flexible enough to be moulded into a circular IVR and how they perform in terms of drug loading and release in the complete form. Future work could also further investigate the blending of PCL with suitable polymers because, as shown herein with PEG (Chapter 2), other polymers can adjust the mechanical properties of the polymer for the further development of IVRs or PCL. As PEG at higher loading lead to increased fragility of the matrices it is recommended that PEG is not likely to be of particular interest

in this regard. Other modifications can be done to increase the flexibility of PCL in the form of coating it with a flexible polymer with delivery windows or the use of PCL in the form of pods with in a flexible ring.

4. *In vivo* activity testing

All tests in this thesis were performed *in vitro.* This included the experimentation to check for PCL leachate toxicity to human vaginal cells and normal commensal vaginal bacteria, and also assays for antibacterial activity of the metronidazole and doxycycline after release from the PCL matrices. Although the cell studies are a cheap and convenient way to check for indications of toxicity, further *in vivo* experimentation is required in order to progress PCL as a polymer for IVRs. The preliminary data presented here can help with deciding future experimental design but does not conclude whether the matrix can be used safely. Additionally, as doxycycline has not previously been delivered through the intravaginal route some basic pharmacokinetic information of drug absorption and safety of doxycycline from the vagina is required. Therefore an animal model is strongly recommended to check the safety and pharmacokinetic behaviour of drug-loaded PCL IVRs. In the literature various models such as sheep, rabbit, pig and macaque monkey are recommended to check the suitability of the vaginal products[15,16] but careful selection of any model is essential to ensure a close correlation between human and animal vaginal anatomical characteristics.

In vivo testing is also required for consideration of ring softness, diameter and flexibility because these are important factors in determining acceptability and adherence to treatment regimes. As the ultimate aim of the IVRs is to make the therapy convenient and acceptable to patients, the optimization of PCL physical characteristics is essential. So, following *in vivo* animal studies, clinical trials would be required to check the suitability, safety and acceptability of PCL IVRs.

5. Conclusion

In this thesis PCL has been proven to be a potential material for the controlled release of antibacterial agents, individually and in combination, for the treatment of STIs as well as for the delivery of macromolecules through the intravaginal route. By manipulating the production method, 100% drug loading has been obtained. This is accompanied by slow release profiles that provide an initial burst release and subsequently maintain daily drug release at a higher level than that required to kill the main causative pathogens

responsible for the diseases. This sustained period of release continued for the full 12 – 14 days of the experiments and was only reduced in length when PEG was added into the polymer matrix, which suggests that it should be possible to create a PCL IVR that is suitable for single insertion at the start of 1 to 2 week duration of therapy. PCL was found to be a safe material for the intravaginal delivery in cell culture experiments, and the soft nature of the polymer makes it likely to be a highly acceptable material to women in terms of comfort for vaginal use. More work need to be done to improve the flexibility of the PCL without compromising the other favourable properties of the polymer. The improvement in design of the PCL IVR by covering it with flexible material or use of PCL as an insert is recommended.

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