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Visualization of the mechanism(s) of drug transport and delivery into and through the nail

WING SIN CHIU

A thesis submitted for the degree for Doctor of Philosophy

University of Bath
Department of Pharmacy and Pharmacology
October 2014

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Declaration

Chapter 2 and Chapter 5: I have collaborated with Dr. Julian Moger and Dr. Natalie Garrett (University of Exeter, UK) for the work on stimulated Raman scattering microscopy and two-photon fluorescence microscopy.

Chapter 7: Some of the experiments were conducted and the data was collected by the project students, Wing Tsun Leung and Wai Han Koh (University of Bath, UK), under my supervision.
**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA</td>
<td>5-aminolevulinic acid</td>
</tr>
<tr>
<td>CARS</td>
<td>Coherent anti-Stokes Raman scattering</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CMT</td>
<td>Critical micellization temperature</td>
</tr>
<tr>
<td>CPX</td>
<td>Ciclopirox</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>FMA</td>
<td>Fluorescein methacrylate</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HPMC</td>
<td>Hydroxypropyl methylcellulose</td>
</tr>
<tr>
<td>IPM</td>
<td>Isopropyl myristate</td>
</tr>
<tr>
<td>KPS</td>
<td>Potassium persulphate</td>
</tr>
<tr>
<td>LSCM</td>
<td>Laser scanning confocal microscopy</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MNs</td>
<td>Microneedles</td>
</tr>
<tr>
<td>MPG</td>
<td>N-(2-mercaptopropionyl) glycine</td>
</tr>
<tr>
<td>MV</td>
<td>Molecular volume</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N-AC</td>
<td>N-acetyl cysteine</td>
</tr>
<tr>
<td>NBC</td>
<td>Nile blue chloride</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>NR</td>
<td>Nile red</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>OMC</td>
<td>Octyl methoxycinnamate</td>
</tr>
<tr>
<td>OPO</td>
<td>Optical parametric oscillator</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEO</td>
<td>Polyethylene oxide</td>
</tr>
<tr>
<td>PG</td>
<td>Propylene glycol</td>
</tr>
<tr>
<td>PF-127</td>
<td>Pluronic F-127</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>P(MMA-co-BMA)</td>
<td>Poly(methyl methacrylate-co-butyl methacrylate)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PPO</td>
<td>Polypropylene oxide</td>
</tr>
<tr>
<td>PSA</td>
<td>Pressure sensitive adhesive</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>SC</td>
<td>Stratum corneum</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SF</td>
<td>Sodium fluorescein</td>
</tr>
<tr>
<td>SRG</td>
<td>Stimulated Raman gain</td>
</tr>
<tr>
<td>SRL</td>
<td>Stimulated Raman loss</td>
</tr>
<tr>
<td>SRS</td>
<td>Stimulated Raman scattering</td>
</tr>
<tr>
<td>T</td>
<td>Penetration depth</td>
</tr>
<tr>
<td>%T</td>
<td>Normalized penetration depth</td>
</tr>
<tr>
<td>TAPP</td>
<td>5,10,15,20-tetakis-(4-aminophenyl)porphyrin</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>$T_{gel}$</td>
<td>Gelation temperature</td>
</tr>
<tr>
<td>TPF</td>
<td>Two photon fluorescence</td>
</tr>
</tbody>
</table>
Abstract

Topical treatment of nail disorders such as onychomycosis and nail psoriasis is desirable to avoid systemic adverse effects and drug-drug interactions. However, the architecture and composition of the nail plate pose a great challenge to the permeation of topically applied drugs. To improve the efficiency of drug permeation into and through the nail, on-going optimization of topical nail formulations is necessary. Presently, to assess drug uptake into the nail post-application of a formulation requires a destructive extraction technique, which provides no detail about the precise localization of the penetrant nor the time course of chemical permeation, and until now, the basic information about the mechanism of drug penetration remains unclear.

The principal aim of this thesis was to demonstrate the novel application of non-destructive imaging techniques, including laser scanning confocal microscopy (LSCM), two-photon fluorescence (TPF) and stimulated Raman scattering (SRS) to study topical nail delivery. The potential use of a number of formulation strategies was investigated based on the insight provided by the imaging tools, including (a) using polymeric particulate-based systems as drug reservoirs, (b) enhancing drug delivery using physical (microneedles) and chemical (penetration enhancers) methods, and (c) development of a thermoresponsive lacquer for topical nail treatment.

SRS microscopy has been successfully used to characterize the concentration-dependent uptake of solvents into the nail. LSCM and TPF imaging have permitted direct visualization of the ‘fate’ of fluorescently-labelled nanoparticles, which remained only at the nail surface and within microneedle-created pores, from which the release of the entrapped model ‘actives’ into the nail occurred. The delivery of an antifungal drug from a sub-micron particle suspension into the nail has also been demonstrated.

Increased nail hydration and pre-treatment with penetration enhancer prior to microneedle-poration improved drug permeation. In addition, a thermoreversible hydrogel has been shown to provide continuous release and delivery of model actives into the nail. This aqueous based lacquer offers an attractive, organic solvent-free, topical nail delivery system with good feasibility and practicability. Finally, intercellular and transcellular penetration pathways were also identified for hydrophilic and lipophilic models, respectively.

Overall, this thesis has provided useful information on the underpinning scientific rationale of the application of different formulation strategies for topical nail delivery upon which further development and optimization might be based.
Chapter 1: Introduction

Overview
The human nail plate is a very efficient physical barrier comprising many layers of dead, keratinized cells which are tightly bound to one another. Nail disorders, especially onychomycosis and nail psoriasis, are very common but unfortunately difficult to cure. Treatment is time-consuming and the relapse rate is high. Although topical medication is simple, attractive and avoids systemic exposure, drug permeation across the nail plate is almost always too low to be effective and long-term oral remedy is usually required. Optimization of topical nail formulations is therefore essential to address this unmet clinical need.

Various enhancement methods to increase nail penetration have been proposed, including physical abrasion, chemical treatment and the use of electric fields. Although there has been some progress, the precise modes of action of the different strategies are not always understood and conflicting results have been reported; furthermore, the exact physicochemical parameters that control nail permeation and transport pathways remain controversial.

The goal of this project is to demonstrate the application of non-destructive imaging techniques, specifically laser scanning confocal microscopy (LSCM), two-photon fluorescence (TPF) microscopy and stimulated Raman scattering (SRS) microscopy, to characterize and determine the mechanisms of a number of novel formulation strategies, including the use of polymeric nanoparticles, microneedle arrays, penetration enhancer and thermogelling formulations.
1.1 The nail

The nail has a highly keratinized structure that forms an efficient shielding cover at the dorsal aspect of the distal or ungual phalanges of the fingers and toes. The functions of the human nail are manifold. It not only protects the delicate fingertips from traumatic injury but also contributes to sensory discrimination (1). It allows the fingers to manipulate small objects in a more refined and dexterous way (2). It is also used for scratching and grooming. Unlike claws and hooves in other mammals, the human nail does not serve as a means of defence and/or attack but is nowadays viewed for more as a cosmetic organ (3).

1.1.1 Structure and anatomy of the nail

The nail apparatus is shown in Figure 1.1 and Figure 1.2. The hard, flat nail plate lies on the highly perfused nail bed. The nail plate is enclosed by the surrounding (lateral and proximal) nail folds and emerges from the underlying nail matrix. The matrix is hidden although the distal portion is sometimes seen as a white, crescent-shaped area, called the lunula, especially on the thumb. The eponychium, also called the cuticle, extends from the proximal nail fold and adheres firmly to the nail plate surface, to prevent the entry of exogenous materials. The hyponychium represents the region underneath the free edge of the nail plate while the distal groove is the cutaneous ridge marking the distal boundary between the nail unit and the finger pulp. The distal margin of the nail bed is observed as a deeper pink (Caucasian) or brown (Afro-Caribbean) transverse band (onychodermal band), indicating the site of ‘seal’ between the nail plate and the nail bed (3-5). The average fingernail growth rate is 3 mm/month and toenail growth is 1 mm/month (1).

Figure 1.1: Schematic diagram showing dorsal and transverse views of the nail. Reproduced with kind permission from (2).
Nail matrix
The nail matrix represents the germinative tissue of the nail apparatus, creating all or most of the nail plate. It is a specialized epithelial structure consisting primarily of keratinocytes which differentiate and migrate towards the surface. During this process, the basal cells become flattened and undergo nuclear fragmentation and condensation of cytoplasm, forming flat, keratinous nail plate cells, known as onychoocytes (4). The direction of nail growth (upward and outward) results from the orientation of the proliferating cells which have their vertical axes aligned diagonally. Unlike the epidermis, the keratinization process in the nail matrix occurs in the absence of a granular layer, and without the formation of keratohyalin. Melanocytes are also present in the nail matrix; although they are usually quiescent, they can become activated and pigment surrounding keratinocytes. The nail matrix can be sub-divided into 3 regions: the dorsal matrix is continuous with the ventral aspect of the proximal nail fold and is responsible for the formation of the outermost dorsal layer; the intermediate (germinative) matrix produces the majority of the nail plate, while the most distal ventral matrix is aligned with the nail bed, contributing to the thin ventral plate (6-9).

Nail fold
The proximal and lateral nail folds enclose more than 75% of the periphery of the nail plate (1). The proximal nail fold is similar to glabrous skin and has two epithelial surfaces, dorsal and ventral. The dorsal aspect is devoid of hair follicles, sebaceous gland and dermatoglyphic markings and the ventral side, being thinner, also lacks rete ridges. At the junction of the two is the eponychium which consists of sheets of overlapping cornified epithelial cells with no nuclei, and serves to protect the nail. The proximal nail fold may contribute to the direction of nail growth by guiding it obliquely over the nail bed (10, 11).
The lateral nail folds are continuous with the adjacent skin at the sides and join medially to the nail bed. Their epidermal structure is comparable to normal skin and they are responsible to the formation of the transverse curvature of the nail plate by compressing it at each side (10).

**Nail bed**

The nail bed extends from the distal margin of the lunula to the hyponychium. It consists of a thin epidermal layer and an underlying dermal part. The epidermal region is only 2-3 cells thick and has no granular layer. The keratinocytes differentiate rapidly to form onychocytes which are incorporated into the deepest (ventral) side of the nail plate. The epidermal layer has a unique pattern of longitudinal ridges which are complementary to the ridging on the under-surface of the nail plate, allowing its strong attachment to the nail bed (1, 2). The dermal region has little fat but no sebaceous or follicular appendages and contains connective tissues that attach directly to the underlying periosteum and the epidermal basal lamina. Within this fibrous network is the vasculature supplying the nail unit, as well as the lymphatics (11).

**Nail plate**

The nail plate is made up of many layers of dead, flattened, keratinized cells which are fused to each other via numerous intercellular links, membrane-coating granules and desmosomes, to form a hard, dense but yet slightly elastic structure. It is not homogenous but comprises three layers: dorsal, intermediate and ventral, with different compositions and properties. Their relative thicknesses have been reported to be 3:5:2 (12).

The outermost dorsal layer contains relatively high calcium, phospholipid and free sulfydryl (SH) groups, but little acid phosphatase activity. The intermediate layer, on the other hand, is richer in stable disulphide bonds but is low in calcium, phospholipid and SH groups. However, it has a high acid phosphatase activity attributed to the retained nuclear remnants. Finally, the thinnest ventral layer is made up of only a few cells layers, having a similar composition to the dorsal nail plate with respect to calcium, phospholipid and SH groups. However, it also shares a high number of disulphide bonds and acid phosphatase activity with the intermediate layer (13).

Keratins belong to the family of intermediate filament forming proteins and are the major components of onychocytes. They can be classified into two types, ‘soft’ epithelial keratins, or ‘hard’ hair-type keratins. The latter comprise a higher number of sulphur-containing amino acids that cross-link the filaments, making them more stable.
and resistant to chemical destruction. Both types of keratins are found in the nail plate, although the majority (80-90%) are the hard keratins. Their location and orientation however differ in the three layers. Using synchrotron X-ray microdiffraction, Garson et al. (8) reported that hair-type keratins are predominantly present in the intermediate layer and are perfectly orientated perpendicular to the growth axis; the epithelial-type keratins, on the other hand, are present in both dorsal and ventral cells, which are dispersed randomly in both perpendicular and parallel directions. Farren et al. (14) suggested that the anisotropic fibre arrangement in the intermediate layer is responsible for the mechanical strength and hardness of the nail, while the isotropic behaviour in dorsal and ventral layers helps to increase the nail’s ability to bend and prevents crack formation.

The lipid content in the nail plate is 0.1-1% w/w, contrasting with the 10% present in stratum corneum. Phospholipids are abundant in the dorsal and ventral layers. They are thought to locate at the cytoplasmic membrane of the onychocytes, filling the ampullar dilations of the dorsal plate and the intercellular spaces in the ventral plate (8). Free fats and long chain fatty acids have also been found although, perhaps of extrinsic origin (7). The low lipid content may contribute to the much higher flux of water (10 times) across the nail relative to that through the skin (15).

The nail plate has a high water content (10-30%) that depends on the relative humidity (RH) of the surroundings. Water acts as a plasticizer, increasing nail elasticity and flexibility (16).

Calcium is the principal metal in the nail plate (0.1% by weight); however, it is believed to contribute insignificantly to the hardness of the nail that is provided by the high sulphur-containing keratins. Other elements have also been detected in small amounts, such as sodium, copper, manganese, zinc and iron (3).

1.2 Embryology of the nail

The first sign of nail development is seen at 8-9 weeks of gestation. A thickened area of epidermis forms an uninterrupted groove, delineating a flattened surface at the end of each digit. This is known as the primary nail field, overlying the tip of the terminal phalanx (1).

At 10-11 weeks, a group of cells arises from the proximal groove of the nail field and grows in the proximal direction, stopping at about 1 mm from the phalanx. This represents the matrix primordium, the dorsal part of which will contribute to the
epithelium of the proximal nail fold, while the ventral part will form the distal and intermediate matrix epithelium. Meanwhile, the distal groove is accentuated by cells formed on the dorsum of the distal tip, the so-called distal ridges.

The primary nail field grows and differentiates, forming the proximal and lateral nail folds by weeks 13-14. The first sign of nail plate growth is also apparent, emerging from beneath the proximal nail fold. At this stage, keratinization in the nail bed epithelium involves the production of keratohyalin from the stratum granulosum. This changes, however, as the nail plate grows over the nail bed at 20 weeks of gestation. The granular layer recedes and the keratinization becomes more parakeratotic (17).

From 20 weeks, the nail plate extends to the distal ridges which now become the hyponychium. A well-demarcated eponychium is also observed and by 32 weeks of gestation, all components of the nail unit are formed.

1.3 Nail diseases and treatments

The nail apparatus suffers from a variety of disorders, comprising about 10% of dermatologic conditions. The most commonly observed include discoloration, thickening, surface abnormalities, nail fold inflammation and nail infections. These affect a patient’s quality of life in numerous ways (18). Two major nail diseases, onychomycosis and psoriasis, and their treatments are now discussed in greater detail.

1.3.1 Onychomycosis

Onychomycosis is a fungal nail infection caused by primarily dermatophytes but also by yeasts and moulds. It has a prevalence rate of 10-40% of the population, increasing with age. The predisposing factors include family history, immunosuppression, diabetes mellitus, trauma to the nails, and damp, enclosed footwear (19). Onychomycosis is classified into 4 clinical categories (19, 20):

**Distal/ lateral subungual onychomycosis (DLSO)** is the most common form which the fungi, usually *Trichophyton rubrum*, invade the nail bed and nail plate proximally from the hyponychium.

**Proximal subungual onychomycosis (PSO)**, also known as proximal white subungual onychomycosis (PWSO), occurs when the fungi, usually *T. rubrum*, invade the nail via the eponychium and the proximal nail fold. The infection spreads distally into the newly formed nail plate and the nail bed. It is relatively uncommon in the general population but occurs frequently in HIV patients.
White superficial onychomycosis (WSO) occurs when the fungi, usually *T. mentagrophytes*, invade the surface of the nail plate directly and later migrate through the nail plate and infect the nail bed. It occurs primarily in the toenails.

*Candida onychomycosis* is caused by *Candida spp.*, mostly *C. albicans* which, unlike the dermatophytes, do not invade nail keratin under normal conditions. Rather, they populate the entire nail plate as a secondary infection in onycholysis (separation of the nail plate from the underlying nail bed) and paronychia (infection of the surrounding nail folds). This is a rare event usually occurring only in patients who are immunocompromised or suffer from chronic mucocandidiasis.

Total dystrophic onychomycosis represents the end stage of nail disease resulting from the progression of any of the four categories above. The nail at this stage is hyperkeratotic and dystrophic.

The treatment of onychomycosis includes nail avulsion (removal of the lesion) and drug-based therapies.

**a) Nail avulsion**

This involves partial or total removal of the nail plate by surgery or using chemicals. Nail surgery is a painful and disfiguring operation that is not generally accepted by patients and is used only in severe nail involvement. Chemical nail removal can be performed and is less painful. Keratolytic compounds, such as urea (at 40%) and salicylic acid can help soften the nail plate, facilitating its detachment from the nail bed (21, 22).

**b) Pharmacotherapy**

Most antifungal drugs, including azoles (the older imidazole, ketoconazole, and the newer triazoles, itraconazole and fluconazole), terbinafine and amorolfine affect the synthesis of ergosterol, a unique sterol cell membrane component in fungi (Figure 1.3). This increases membrane permeability of the fungi, leading to leakage of cellular contents and lysis. Alternatively, griseofulvin is active against growing hyphae, inhibits nucleic acid synthesis and inhibits fungal cell mitosis (20). Ciclopirox acts as a chelating agent, inhibiting iron-dependent mitochondrial enzymes and electron transport-related processes. This leads to reduced energy production, accumulation of toxic peroxide, reduced uptake of nutrients, and inhibits synthesis of proteins and nucleic acids (23).
Figure 1.3: Ergosterol biosynthetic pathways and inhibition by allylamines, azoles and morpholines. Modified from (24).

Table 1.1 summarizes the recommended dosage regimens and the reported peak nail concentrations achieved with different antifungals, as well as their minimum inhibitory concentrations (MIC) against the common dermatophytes in onychomycosis. Topical therapies appear to deliver higher drug concentrations within the nail (well above the MIC ranges) than oral treatments. However, both topical and systemic treatments are limited by long treatment duration because (a) drug concentration in the nail plate is often not achieved after, at least, a couple of weeks and (b) nail cure is not obtained before months. Furthermore, relapses and re-infections are common irrespective of the types of treatments employed.
Table 1.1: Dosage regimens, minimum inhibitory concentrations (MIC) and peak nail concentrations of various antifungals.

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Dosage regimen</th>
<th>MIC (µg/mL)</th>
<th>Peak in vivo nail plate concentrations (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Griseofulvin</td>
<td>500 mg/d - 1 g/d until recovery</td>
<td>0.5-2</td>
<td>N/A</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>200-400 mg/d until recovery</td>
<td>0.03-16</td>
<td>N/A</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>200 mg/d for 3 months or 400 mg/d for 7 days and repeat after 21-day interval</td>
<td>0.06-32</td>
<td>0.6-0.9 (200 mg/d for 3 months)</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>150-450 mg once weekly for 3 months (fingernails) or 6-9 months (toenails)</td>
<td>1-64</td>
<td>2.1 (fingernail) 9.6 (toenail) (150 mg/w for 4 weeks)</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>250 mg/d for 6 weeks - 3 months (longer for toenail infections)</td>
<td>0.003-2</td>
<td>0.39 (250 mg/d for 4 weeks)</td>
</tr>
<tr>
<td>Amorolfine</td>
<td>1-2 topical applications/week</td>
<td>0.01-0.08</td>
<td>640 (2 applications/week for 15 days)</td>
</tr>
<tr>
<td>Ciclopirox</td>
<td>Once daily topical application</td>
<td>0.015-0.125</td>
<td>1780 (1 application/d for 14 days)</td>
</tr>
</tbody>
</table>

* Dosing regimens referenced from BNF (25) except for fluconazole (26) and ciclopirox (23), which are not licensed in the UK.
* In vitro MIC ranges against dermatophyte species (27-30).
* Peak nail concentrations (23, 31-34).

Systemic treatments

A systemically administered drug can migrate from the capillary system and penetrate the keratinized nail plate and the nail matrix via diffusion from the nail bed (31).

Griseofulvin was the only available oral treatment before the introduction of the newer antifungals and is only active against dermatophytes. Prolonged treatment that may last for more than a year is required for maximal recovery due to the drug’s poor persistence in the nail plate at a therapeutic level after oral dosing. Patient compliance is poor, with a cure rate of only 3-38%, and relapse is common (20). Nevertheless, the drug is generally well tolerated and the most common side effects are hypersensitivity (skin rash, urticaria), headache and gastrointestinal disturbance. However, griseofulvin is also teratogenic and may induce the metabolism of other drugs, including anticoagulants (decreased activity) and oral contraceptives (reduced efficacy) (25).

Ketoconazole was the first marketed orally administered imidazole and is active against Candida as well as dermatophytes. The duration of treatment is similar to griseofulvin but its use is limited by the occurrence of side effects and drug interactions (1). Although life-threatening hepatotoxicity has been reported to occur very rarely, the risk is greater with long-term administration and liver function has to be monitored. Being a
potent cytochrome P-450 (CYP3A4) inhibitor, ketoconazole can reduce the metabolism of a range of co-administered drugs including, for example, anti-arrhythmics, anticoagulants, simvastatin and ciclosporin (25).

**Itraconazole** is a triazole antifungal, active against dermatophytes, yeasts and other pathogenic moulds. It has been reported to penetrate the nail plate more quickly (within 2 weeks of the start of treatment) and to have a long persistence time. As for ketoconazole, hepatotoxicity is very rarely reported with this drug. Itraconazole can also have significant negative inotropic effects and therefore should not be given to patients with ventricular dysfunction or a history of heart failure. The drug also inhibits CYP3A4 (22).

**Fluconazole** is another broad-spectrum triazole antifungal. It appears to penetrate the nail as well as itraconazole (1). The drug has a better adverse effect profile but inhibits both CYP3A4 and CPY2C9 in a dose-dependent manner; as a result, a number of drugs metabolized via these pathways are contraindicated or require close monitoring.

**Terbinafine** is an allylamine antifungal which specifically inhibits squalene epoxidase in the ergosterol synthesis pathway. It is effective against a wide range of pathogenic fungi at relatively low concentrations. The drug is generally well tolerated and the most common adverse effects involve gastrointestinal discomfort and nausea. Roberts (27) compared the clinical outcome of terbinafine treatment with that of other oral antifungals and reported that the former was the most cost-effective, and provided the highest efficacy (cure rate of 70-80%).

**Topical treatments**

Amorolfine and ciclopirox are the currently available antifungals for topical treatment of onychomycosis.

**Amorolfine** shows strong fungicidal activity against a broad spectrum of pathogenic fungi. It is applied as a 5% w/w lacquer. Tolerable, local side effects, such as a burning sensation, contact dermatitis, redness, irritation and pain are associated with its use. In a large clinical study (35) involving 714 patients having mild infection of the nail (with intact lunula or matrix), mycological cure (negative culture and microscopy) was achieved in 52.1% and 64.3% of the toenail and fingernail, respectively. The number of reported adverse effects was less than 1%.

**Ciclopirox** also exhibits a strong and broad fungicidal activity against the whole spectrum of fungal pathogens. It is applied as a 8% w/w lacquer. The adverse effect profile is similar to that of amorolfine. The mycological cure rates in the worldwide
clinical studies were 29-36% (US) and 46.7-85.7% (non-US) and a good safety profile was reported (36).

Table 1.2 lists some examples of amorolfine and ciclopirox nail lacquers licensed in Europe. The current rationale for topical monotherapy is, however, restricted to early infections which involve only the distal 2/3 of the nail plate of up to 2-3 digits or to prevent reinfection (22). Oral medication and/or combination therapy (oral and topical) is usually required to achieve higher cure rates.

Table 1.2: Some examples of amorolfine and ciclopirox nail lacquers licensed in Europe for the treatment of onychomycosis.

<table>
<thead>
<tr>
<th>Active ingredient</th>
<th>Strength (w/w)</th>
<th>Brand name</th>
<th>Recommended dosage frequency</th>
<th>List of excipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amorolfine</td>
<td>5%</td>
<td>Loceryl®</td>
<td>Once or twice weekly application</td>
<td>Ammonio methacrylate copolymer, triacetin, butyl acetate, ethyl acetate, ethanol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curanail®</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Omicur®</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciclopirox</td>
<td>8%</td>
<td>Batrafen®</td>
<td>Apply once every other day for the first month, reduced to at least twice weekly in the second month then once weekly from the third month onwards</td>
<td>Butyl ester of methyl vinyl ether/maleic anhydride copolymer, isopropyl alcohol, ethyl acetate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciclochem®</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciclopoli®</td>
<td>Once daily application</td>
<td>Hydroxypropyl chitosan, cetylstearyl alcohol, ethyl acetate, ethanol</td>
</tr>
</tbody>
</table>

In addition to amorolfine and ciclopirox lacquers, the topical therapy market has grown rapidly in recent years and a wide range of additional products is currently commercialized. Table 1.3 lists some of the examples available in the UK. The cost of these treatments is not trivial given that multiple purchases are required for the typically lengthy treatments involved. In addition to the use of urea (based on its chaotropic action), most of these products are acidic in nature, anticipating that low pH produces a fungistatic or even fungicidal activity. This strategy probably has its origin in older home remedies and the use of vinegar (5% acetic acid), for example, to treat fungal nail infections (37). Despite the high efficacy claimed (advertised as killing 99.9% of
pathogenic germs), these treatments have not been rigorously tested in clinical trials. The mode of action is unclear and strong scientific support is also lacking. Finally, the antiseptic agent, benzalkonium chloride, has also been formulated for the treatment of nail disease, but almost certainly suffers from the ubiquitous problem of poor permeation and sub-therapeutic concentrations in the nail matrix or nail bed.

Table 1.3: Some of the newer topical products available in the UK for the management of fungal nail infection.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Price a</th>
<th>Main ingredients b</th>
<th>Claims b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scholl fungal nail treatment</td>
<td>£19.40 /3.8 mL</td>
<td>Undisclosed</td>
<td>Create a hostile environment for the fungus by lowering the nail pH</td>
</tr>
<tr>
<td>Excilor®</td>
<td>(brush) £19.99 /3.3 mL (pen) £22.99 /4 mL</td>
<td>Ethyl lactate, water, acetic acid, penetration enhancer, film-forming agent</td>
<td></td>
</tr>
<tr>
<td>Nailner®</td>
<td>(brush) £19.99 /5 mL (pen) £19.99 /4 mL</td>
<td>Ethyl lactate, water, lactic acid</td>
<td>Soften and remove the infected nail</td>
</tr>
<tr>
<td>Canespro®</td>
<td>£29.99 /10 g</td>
<td>Urea 40% ointment, Waterproof plasters</td>
<td></td>
</tr>
<tr>
<td>ClearZal®</td>
<td>£21.49 / 30 mL</td>
<td>Benzalkonium chloride, water</td>
<td>Antimicrobial action</td>
</tr>
</tbody>
</table>

a Market price based on (38).

b,c According to patient information leaflet or product packaging.

1.3.2 Nail psoriasis

Nail psoriasis is a chronic condition affecting 1-3% of the population and has a lifetime incidence of 80-90% in individuals with skin psoriasis (16). The disease results from an abnormal immunological T-cell activated proliferation and differentiation of keratinocytes. Clinical manifestation depends on the region of the nail unit affected. Pitting (visible holes in the uppermost layers of the nail plate) is the most common sign, indicating nail matrix involvement. Onycholysis, subungual hyperkeratosis (build-up of chalk-like materials under the nail plate) and splinter haemorrhages (red or brown longitudinal bands due to rupture of the delicate capillaries in the nail bed) are typical of psoriasis of the nail bed and hyponychium. Nail discoloration (‘oil-drop’ discoloration or salmon-coloured areas) also occurs if the nail bed and, sometimes, the distal nail matrix are affected (2, 19).

Nail psoriasis is difficult to cure and refractory to treatment. Even now, a standard therapeutic regimen does not exist and treatment options depend on the type of lesion and the patient’s preference. Disease management approaches can be divided into systemic, topical, intralesional and radiation therapies.
**Systemic treatments**

Antipsoriatic drugs include immunosuppressants (ciclosporin, methotrexate) and retinoids (acitretin). Their efficacy in the treatment of skin psoriasis is evident but little is known of their efficacy on the nail. Their use is nonetheless reserved for recalcitrant or in extensive disease because of their significant adverse effects (immunosuppressive and teratogenic) and drug interactions (2).

**Topical treatments**

Topical treatment of nail psoriasis is less well documented than of the skin. Topical medications licensed for the treatment of skin psoriasis are commonly used off-label for the management of nail psoriasis. Formulations, such as creams, ointments, gels, lotions or lacquers, are applied to the nail plate, the nail folds and/or as close to the nail bed as possible by trimming the nail back to the point of cleavage from the nail bed. Occlusion is usually recommended and urea and salicylic acid are sometimes co-formulated with the drug to maximize permeation (3). Some commonly used topical antipsoriatic drugs are as follows:

- **Corticosteroid** (e.g., betamethasone, clobetasol, fluocinolone and triamcinolone) and **vitamin D₃ analogues** (calcipotriol, tacalcitol and calcitriol) are the most popular, having anti-inflammatory, antiproliferative and immunosuppressive activities. Clinically, improvement of subungual hyperkeratosis by reducing the nail thickness has been reported (39).

- **5-fluourouracil** inhibits DNA synthesis and has been suggested for reducing pitting and nail thickening conditions. The use of this drug should, however, be avoided in onycholysis which is exacerbated by the treatment (40).

- **Anthralin** decreases inflammation, increases cellular differentiation and reduces proliferation in psoriasis. It provides moderate improvement in psoriatic dystrophies especially in cases of nail bed involvement. However, the drug is less effective in matrix psoriasis (the source of pitting), probably due to its poor penetration into the underlying nail matrix. It is nevertheless a safe option with no known systemic side effects (but is not particularly popular as it stains the skin and clothing).

- **Tazarotene** is a topical retinoid which acts by regulating the differentiation and proliferation of keratinocytes, and controls the inflammation. It reduces onycholysis and improves nail pitting. Local side effects, including peeling and erythema of the proximal nail folds, have been reported (41).
Ciclosporin may also be used topically. Pitting and onycholysis have been cleared after use of the drug in an oily formulation. Further research into the topical use of other immunosuppressants, such as tacrolimus and pimecrolimus, may offer more alternative options in the future (2).

**Intralesional injections**

Small doses of corticosteroids (primarily triamcinolone) can be injected directly into or near the nail unit and are particularly effective in treating lesions within the nail matrix. The proximal nail fold is the optimal and least painful site of injection. Deeper matrix and nail bed injections may be required for conditions such as onycholysis, subungual hyperkeratosis and nail discolouration, but these are very painful and local anaesthesia is essential. While subungual hematomas may occur following intralesional injection, skin atrophy, and more severely, atrophy of the underlying terminal phalanx, collagen and extensor tendon, can result from the repeated treatment with steroids (2, 40).

**Radiation therapies**

Treatments can be classified as phototherapy or photochemotherapy. The former involves the use of ultraviolet (UV) radiation, mostly UVB (\(\lambda = 290-320\) nm) and narrowband UVB (\(\lambda = 313\) nm). Photochemotherapy, or PUVA, couples the use of UVA (\(\lambda = 320-400\) nm) with oral or topical psoralen, a light-activated skin sensitizer. Radiation therapy has been validated for psoriatic skin treatment but not for the nail. Their mechanism of action is not fully understood but is probably related to suppression of an overactive immune response. The approach is believed to be useful in dystrophies arising from the nail bed but not from the matrix. Other types of radiotherapy, including superficial radiotherapy (SRT), Grenz ray therapy and electron beam therapy, have demonstrated variable efficacy. Potentially serious adverse reactions, such as decreased blood flow and a risk of malignancy, may limit the use of radiation in the treatment of nail psoriasis and further research in this area is required (2).

**1.4 Topical drug delivery to the nail**

Transungual delivery involves the uptake of drug incorporated in a topical formulation into the nail plate and its subsequent diffusion into the deeper tissues, possibly reaching the nail bed (Figure 1.4).
Figure 1.4: Transungual delivery and key factors that determine its efficacy.

Before entering the nail plate, significant pre-absorptive loss of drug due to routine activities is ideally minimized (42). Formulations, such as nail lacquers and protective plasters can provide good adherence to the nail surface and lengthen drug-nail contact time (16). However, the amount of drug available for diffusion can be severely compromised by crystallization when the typically volatile alcohol (a good solvent for many drugs administered as nail lacquer) evaporates rapidly after application. Although this may lead to transient supersaturation, this metastable state quickly dissipates and most of the administered drug precipitates as a solid (and no longer in a diffusible form).

Once in the nail plate, drug transport within and across the keratin network is influenced by many factors. The properties and the conditions of the nail can play an important controlling role that can be affected by certain formulation components. The hydrated nail plate has been suggested to behave more like a hydrogel (a network of hydrophilic polymer chains) than a lipophilic membrane such as that of stratum corneum (43). The penetration of solutes through the nail plate has also been described (44) as the passive diffusion of substances across a homogenous polymer network (45), involving a ‘free volume mechanism’. In this description, thermal movement of the polymer chains creates ‘spaces’ or holes, which can be occupied sequentially by permeating molecules as they transverse the network. The penetration rate is limited by the formation frequency and the free volume of the holes as determined by the temperature, the nature of the polymer, and its interaction with the permeant. The theory clearly implies the influence of size and shape of the diffusing molecules on the transport kinetics. Drug uptake into the nail plate also depends on its ability to partition into the keratin network and depends therefore on the drug’s hydrophilicity/lipophilicity and its ionization state (46).
If the nail plate is treated as a homogenous membrane, passive diffusion of drug in steady state can be most simply described by Fick’s 1st law of diffusion (Eq 1.1), where an infinite applied dose and a downstream perfect sink are assumed:

\[
J = \left( \frac{D_m K_m}{h} \right) \cdot C_v = k_p \cdot C_v \quad \text{(Eq. 1.1)}
\]

where \( J \) is drug flux (amount per unit area per unit time), \( D_m \) is the diffusion coefficient of drug in the membrane, \( K_m \) is its membrane/vehicle partition coefficient, and \( h \) is the diffusion path length. \( C_v \) is the drug concentration in the vehicle and \( k_p \) is the permeability coefficient (\( = \frac{D_m K_m}{h} \)). Drug transport across the nail can therefore be influenced by various factors determining any of these parameters, which can also be altered or enhanced by the formulation.

### 1.4.1 Factors affecting drug permeability across the nail plate

**Nail properties**

Drug flux across the nail plate decreases with increasing nail thickness. An inverse relationship between the flux of 5-fluorouracil (5-FU) and the thickness of healthy and diseased nail plate has been demonstrated (47) (Figure 1.5). The authors suggested that the permeability through healthy and fungal nail plates was not significantly different although heavily infected nails were excluded from the study because of the uneven thickness measured, which possibly might produce higher flux due to nail destruction by the fungi.

![Figure 1.5: (a) 5-FU flux across the healthy nail plate as a function of nail thickness (h). (b) 5-FU fluxes across healthy (□) and diseased nail plates (●) as a function of the reciprocal of the nail plate thickness (1/h). Reproduced with kind permission from (47).](image-url)
The degree of hydration of the nail plate is another factor influencing drug permeability. It has been documented thathydration ‘loosens’ nail structure by altering the van der Waal’s forces, hydrogen bonding and ionic interactions within the keratin matrix, making it more elastic and presumably more permeable to the diffusing molecules (48-51). An increase in ketoconazole flux through the nail plate with increasing relative humidity (RH) on the dorsal side, which in turn increases nail hydration has been reported (50) (Figure 1.6). The greatest increase was observed at the region of 80-100% RH. It was deduced that the drug diffusivity increased with increasing water in the nail plate, similarly to its behaviour in swollen polymeric hydrogels. This is consistent with the ‘free volume theory’ and that the plasticizing effect of water increases the segmental mobility of the polymer chains within the nail structure.

![Figure 1.6: Steady state fluxes \( J_{ss} \) of ketoconazole through human fingernails as a function of relative humidity. Reproduced with kind permission from (50).](image)

**Molecular size of penetrant**

An inverse relationship between permeant diffusivity \( D_m \) within the nail and its molecular size/weight is anticipated by the theories of Cohen & Turnbull (52) and Lieb & Stein (45).

### Cohen & Turnbull
\[
D_m = D_0 \cdot e^{-\beta' \cdot MV} \quad \text{(Eq. 1.2)}
\]

Substituting into \( k_p = \frac{D_m K_m}{h} \) (Eq 1.1) and taking logarithms:

\[
\log k_p = \log \frac{D_0}{h} - \beta' \cdot MV + \log K_m \quad \text{(Eq. 1.4)}
\]

\[
\therefore \log k_p \approx k - \beta' \cdot MW \quad \text{(Eq. 1.6)}
\]

### Lieb & Stein
\[
D_m = D_0 \cdot MW^{-z} \quad \text{(Eq. 1.3)}
\]

\[
\log k_p = \log \frac{D_0}{h} - z \cdot \log MW + \log K_m \quad \text{(Eq. 1.5)}
\]

\[
\therefore \log k_p \approx k - z \cdot log MW \quad \text{(Eq. 1.7)}
\]
D\textsubscript{0} represents the diffusion coefficient of a hypothetical molecule having zero molecular volume (MV), MW is molecular weight, k\textsubscript{p} is the permeability coefficient. β, z and k are constants, and β’ equals β/2.303. β” also includes a conversion factor for the substitution of MW for MV (53). The permeability coefficients of a series of alkyl nicotinates as well as other substances decrease across human nail plate and bovine hoof membrane with increasing molecular size/weight (Figure 1.7). The permeability of the nail plate was twice as sensitive to changes in MW as that of hoof membrane, suggesting a more tortuous diffusion path in the denser keratin fibre network of the nail (44). A similar trend in the nail permeation of a range of drugs has also been observed (47).

Despite the established relationship, a clear cut-off above which compounds become unable to permeate the nail plate has not been confirmed. While Mertin and Lippold (44) demonstrated the permeation of compounds with MW ranging from 151.2 to 777.1, Kobayashi et al. (47) were unable to determine the penetration of drug having a MW above 240.

**Figure 1.7:** Logarithms of the product of diffusivity (D\textsubscript{m}) and partition coefficient (K\textsubscript{m}) of chemicals across the nail plate (Δ, △) and the bovine hoof membrane (○, ●) against the (a) MW or (b) log MW. D\textsubscript{m}, K\textsubscript{m} expressed in cm\textsuperscript{2} s\textsuperscript{-1}. (Δ, ○) methyl, ethyl, butyl, hexyl and octyl nicotinates; (△, ●) other substances (paracetamol, phenacetin, diprophylline, chloramphenicol, iopamidol). Reproduced with kind permission from (44).

**Hydrophilicity/ lipophilicity of penetrating compound**

The effect of drug lipophilicity on nail permeability remains controversial. As the nail plate is generally considered to behave as a hydrophilic gel membrane, the unfavourable permeation of hydrophobic drugs is expected. However, the existence of a parallel lipid pathway has been suggested (43) based on a study of a series of homologous alkanols (C\textsubscript{1}-C\textsubscript{12}) (Figure 1.8a). In contrast to the positive correlation observed in parallel
experiments on skin (Figure 1.8b), permeability through the nail showed the opposite trend from water to octanol. This was explained by the hydrophilic nature of the nail plate, and decreased partitioning into the keratin matrix with increasing hydrophobicity. At very high lipophilicity (C_{10} and C_{12}), however, the appearance of a minor lipid route was proposed to account for the upturn in transport of these higher alkanols. The role of this specific pathway for very hydrophobic compounds was further illustrated by the decreased permeation of decanol when the nail plate was delipidized whereas those of the lower chain length homologous were increased (Figure 1.8a) (54).

Figure 1.8: Permeabilities of n-alkanols from dilute aqueous solution through (a) normal (●) and delipidized nail plate (■), and (b) human epidermis. Re-plotted from (43), (54) and (55), respectively.

In contrast, subsequent report on the penetration of nicotinic acid esters (56) (Figure 1.9a) and p-hydroxybenzoic acid esters (47) (Figure 1.9b), failed to confirm the existence of this lipophilic pathway. Very little dependence of the permeabilities of these compounds on lipophilicity was observed. Again, the contrast between the hydrophilic nail plate and the lipophilic skin barrier was clearly apparent (Figure 1.9c).

Figure 1.9: Dependence of permeability on lipophilicity (as expressed by the Log octanol-water partition coefficient) (Log P) for (a) nicotinic acid esters across bovine hoof membrane (●) and human nail plate (■), (b) hydroxybenzoic acid esters across the nail plate, and (c) nicotinic acid esters across human skin in vivo. Reproduced with kind permissions from (56), (47) and (57), respectively.
Ionization

Conflicting results have been reported on the influence of drug ionization on nail permeation. The penetration of drugs in charged and uncharged forms across the nail plate has been addressed (47) and indicated that the former were less permeable, and that molecular weight (MW) was more important. It was suggested that hydration of the ionized drug increased the apparent MW, and thereby lowered the permeability. It was also shown (56) that undissociated benzoic acid and pyridine penetrated bovine hoof membrane to a greater extent than their ionized forms. It was postulated that electrostatic repulsion between the charged keratin membrane and the like-charged diffusing molecules explained these findings. The results of these two studies are summarized in Table 1.4. Although similar conclusion has been drawn from other work (58), Walters et al. (59) found that the permeability coefficient of miconazole, a weakly basic drug (pKa = 6.65), was essentially the same in both dissociated and undissociated states (at pH ranges from 3.1 to 8.2) (Figure 1.10). The exact impact of ionization is unclear, therefore, and it is difficult to see how this parameter might usefully be exploited from a formulation standpoint in a practical way.

Table 1.4: Permeability coefficients of ionizable drugs at different pH through bovine hoof membrane and human nail plate.

<table>
<thead>
<tr>
<th>Drug</th>
<th>pKa</th>
<th>pH</th>
<th>Ionization</th>
<th>Permeability coefficient (Kp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoic acid</td>
<td>4.19</td>
<td>2.0</td>
<td>Unionized</td>
<td>78.62 ± 16.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.4</td>
<td>Ionized</td>
<td>8.29 ± 1.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.21</td>
<td>Unionized</td>
<td>12.84 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.12</td>
<td>Ionized</td>
<td>0.91 ± 0.136&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.19</td>
<td>2.0</td>
<td>Ionized</td>
<td>19.14 ± 7.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.4</td>
<td>Unionized</td>
<td>44.80 ± 10.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>7.92</td>
<td>4.33</td>
<td>Ionized</td>
<td>0.031 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.21</td>
<td>Unionized</td>
<td>0.39 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>K<sub>p</sub> (x10<sup>8</sup> cm<sup>2</sup> s<sup>-1</sup>) across bovine hoof membrane. Adapted from (56).

<sup>b</sup>K<sub>p</sub> (x10<sup>7</sup> cm s<sup>-1</sup>) across human nail plate. Adapted from (47).

![Figure 1.10: pH-permeation profile for miconazole across human nail plate. Figures beneath the curve are % dissociations. Reproduced with kind permission from (59).]
**Effect of vehicles**

The facilitating role of aqueous vehicles on drug permeation into and through the nail has been suggested. For example, a five-fold increase in the nail permeability of a series of homologous alcohols was observed (54) when applied diluted in water as compared to the neat liquids. This was explained by reduced nail swelling when the pure alcohols were administered and the lower free volume available between the keratin fibres. It was also demonstrated that dilution with dimethyl sulphoxide (rather than water) reduced the nail permeation of methanol and hexanol, again pointing to the unique hydrating properties of water in the nail and the concomitant effect on permeability.

The effect of lipophilic vehicles on drug permeation has also been studied. The penetration of chloramphenicol from a phosphate buffered solution was compared with that from n-octanol and medium-chain triglycerides across hoof membrane and human nail plate (60). The drug was saturated in all solvents yet, despite the different solubilities, there was no significant difference between the fluxes observed (Table 1.5). In this case, despite the potential enhancement upon hydration, the maintenance of maximum thermodynamic activity of the drug in the different formulations was the dominant factor.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Solubility (g L(^{-1}))</th>
<th>(10^4 J_{\text{max}}) (ng cm(^{-2}) h(^{-1})) Hoof membrane</th>
<th>(10^4 J_{\text{max}}) (ng cm(^{-2}) h(^{-1})) Nail plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer, pH 7.4</td>
<td>4.52 ± 0.064</td>
<td>11.31 ± 3.28</td>
<td>2.28 ± 0.59</td>
</tr>
<tr>
<td>n-Octanol</td>
<td>23.21 ± 0.767</td>
<td>9.44 ± 1.89</td>
<td>2.54 ± 0.18</td>
</tr>
<tr>
<td>Medium-chain triglycerides</td>
<td>2.35 ± 0.013</td>
<td>11.28 ± 2.78</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

**1.5 Current and novel strategies for transungual delivery**

To successfully treat nail disorders, drugs applied topically should permeate deep into the nail plate and reach to the nail bed and nail matrix. To tackle the poor permeability of the nail plate, different strategies have been proposed to optimize topical formulations, to modify the structure of the nail barrier and to drive drug permeation using physical methods.
1.5.1 Formulation optimization

Nail lacquers

Currently, medicated nail lacquers are the most commonly used transungual delivery systems for the treatment of onychomycosis. Conventional nail lacquer contains essentially an organic solution of drug, a film-forming polymer and a plasticizer which increases the flexibility and durability of the coating. In principle, when the lacquer solution is applied on the nail, the volatile solvent evaporates, leaving a hard, water-resistant film in which the drug is now present at a high concentration and from which it can be slowly released and penetrate into the nail. This occlusive film formed on the surface also increases hydration of the upper nail layers and may facilitate drug permeation as discussed previously.

To penetrate into the nail plate, the drug must be released from the polymer film which may be thought of as a matrix-type controlled-release device. After solvent evaporation, the drug is either dissolved or dispersed in the polymer although only the dissolved form can be released, and provide the driving force for subsequent diffusion (46).

The permeation of chloramphenicol from Eudragit RL, poly (methyl methacrylate)-based lacquers (Figure 1.11a) and the uptake of amorolfine from Loceryl® nail lacquer (Figure 1.11b) both increased with increasing applied drug concentration (60, 61).

![Figure 1.11](image)

Figure 1.11: (a) Penetration of chloramphenicol through the hoof membrane from Eudragit RL lacquers containing different drug concentrations, 2.2% (●), 18.5% (■), 31.3% (▲) and 47.6% (◆). (b) Amount of amorolfine taken up into porcine hoof horn as a function of drug concentration in Loceryl® nail lacquer. Reproduced with kind permissions from (60) and (61), respectively.

The nail delivery of a ciclopirox gel formulation (2% w/v) containing propylene glycol and urea, a marketed gel (0.77% w/v) and a commercialized lacquer (8% w/v) containing water-insoluble, film-forming polymer have been compared (62). After 14 days of application, the marketed gel, despite containing the lowest ciclopirox
concentration, appeared superior to the other two formulations. A significantly higher amount of unabsorbed drug was recovered on the nail surface following application of the lacquer. This has prompted more research into the use of aqueous-based nail lacquers. For example, an experimental formulation based on the water-soluble, film-forming agent, hydroxypropyl chitosan (HPCH), delivered ciclopirox across bovine hoof membranes as well as that from the marketed water-insoluble lacquer (Penlac®) (63). Both formulations contained 8% w/w drug and achieved similar fluxes of ciclopirox at steady state; however a much shorter lag time was observed for the chitosan-based lacquer. Subsequently (64), it was shown that the same lacquer containing amorolfine was superior to the market reference (Loceryl®). Based on these results, Ciclopoli®, a water-soluble, chitosan-based nail lacquer containing ciclopirox, has been marketed recently in some countries. However, more frequent applications are required when using water-soluble polymers because the films are easily removed by simple washing. In response, a bilayered nail lacquer has been investigated (65) which consisting of an underlying drug-loaded hydrophilic hydroxypropyl methylcellulose (HPMC) layer with polyethylene glycol (PEG 400) as an enhancer, and an external hydrophobic vinyl polymer coating (Figure 1.12a). The occlusive outer layer not only increased the uptake of terbinafine into and its permeation across the nail plate (probably by augmenting the hydration level of the keratin network), but also provided the lacquer with resistance to multiple washings (Figure 1.12b).

Figure 1.12: (a) Cross-section of the nail plate (A) treated with a bilayered nail lacquer, comprising a drug-loaded hydrophilic layer (B) and an external hydrophobic vinyl polymer layer (C). (b) Cumulative amounts of terbinafine lost from the control hydrophilic lacquer devoid of PEG 400 (grey column), the hydrophilic monolayer lacquer (white column) and the bilayered lacquer (black column) during multiple washings. Reproduced with kind permission from (65).

Nail patches
A transungual drug delivery patch consists of a drug-impermeable backing layer, a drug-loaded pressure sensitive adhesive (PSA) and a release liner (66) providing a
simple and convenient system with good adhesion that requires less frequent applications. The permeation of ciclopirox across porcine hoof membrane from various PSA has been investigated (66). An acrylic adhesive with a hydroxyl functional groups (AA-OH) produced the highest delivery. However, the drug crystallized in all matrices except one suggesting the need for additional work to improve the formulation approach. Donnelly et al. (67) formulated a bioadhesive patch containing 5-aminolevulinic acid (ALA), a photosensitizer that has bactericidal and fungicidal activity upon radiation. The patch comprised of poly(methylvinylether/ maleic anhydride) (PMVE/MA), tripropylene glycol methyl ether (TPM) and ethanol, and was loaded with 50 mg/cm² ALA. Being small and polar, ALA penetrated reasonably well into and across the nail plate. However, the maximum ALA concentration achieved at the inner nail was less than one-tenth of that required to produce a 90% reduction in the viability of C. albicans and T. interdigitale, respectively. It appears, therefore, that further steps are necessary to improve the ALA penetration, perhaps by filing off some of the nail plate or by the use of penetration enhancers. Alternatively, because of the potential for toxicity with high level of ALA concentrations, the incorporation of iron chelators to enhance photosensitization may need to be considered to allow a lower ALA dose to be used.

1.5.2 Nail modification

Transungual permeation may also be enhanced by underpinning the barrier properties of the nail plate using physical abrasion and/or chemical treatment.

A) Physical enhancement

Filing

Filing the nail plate surface is the simplest way to reduce the barrier thickness and possibly remove part of the infected area; the procedure is often recommended before the application of topical formulation. The increased permeation of 5-FU and flubiprofen across the nail plate, which had been filed, has been demonstrated (12).

Etching

Acid-etching has been suggested for roughening the surface of the nail, and increasing the available surface area for transport. A distinct change in topography is apparent when the nail surface is treated with 10% phosphoric acid (68) (Figure 1.13) and this results in increased bioadhesion of the ketoconazole-loaded polymeric films as well as significantly increased drug permeation. It is proposed that etching reduces the effective
nail thickness and increases the microporosities within the barrier, facilitating thereby diffusion of drug from the polymeric system.

![Figure 1.13: Atomic force microscopy (AFM) images of the dorsal surfaces of (a) non-etched, and (b) etched nail plate. Reproduced with kind permission from (68).](image)

**Microporation**

The penetration pathways across the compact nail structure might also be shortened or by-passed by microporation of the nail plate, for example using an ultra-short laser pulse (69). The laser energy is absorbed by water or protein in the nail plate, heating up and melting the material, leading to vaporization and thermo-mechanical damage. Using ultra-short pulses, the degree of destruction can be controlled to produce small craters (300-400 μm) on the nail plate but avoiding cracking or collateral damage (Figure 1.14a).

A hand-held device (PathFormer, manufactured by Path Scientific, Carlisle, MA, USA), has also been developed to drill microconduits through the nail plate (Figure 1.14b). By monitoring the electrical resistance of the nail at the site of treatment, it is possible to create holes of a controlled depth without penetrating the nail bed. This procedure was shown to be well tolerated, causing minimal pain and discomfort in a small clinical study, and it has been suggested for nail trephination in the treatment of subungual hematomas (70).
Figure 1.14: (a) Scanning electron microscopy (SEM) image of a crater formed on the nail plate by the application of ultra-short pulse laser (350 fs at 10Hz). (b) Photography of a hole drilled on the nail plate using the PathFormer device. Reproduced with kind permissions from (69) and (70), respectively.

B) Chemical enhancement

The use of chemical agents, which can interact with the nail keratin network and destabilize its integrity, is a strategy that can improve drug permeation. Some examples are discussed below.

Keratolytic agents

Keratolytic agents, such as urea and salicylic acid can be used to soften the nail plate and facilitate its detachment and chemical dissolution perhaps by weakening the intermolecular bonds and disrupting secondary and tertiary keratin structure, to cause protein unfolding (71). A pronounced fracture of the nail surface treated with keratolytic substances has been demonstrated and the formation of pores in the keratin matrix was hypothesized (72). However, despite these structural modifications, incorporation of urea (40%) into an antifungal formulation and pre-treatment with 20% salicylic acid for 10 days failed to promote drug transport and no permeation through the nail was detected over 60 days. Equally, neither urea nor salicylic acid, both used at 20%, were able to enhance water transport through the nail plate (71).

Reducing agents

Compounds with free sulfhydryl group (-SH) may destabilize the nail keratin network by reducing disulphide (S-S) linkages (16):

$$\text{Keratin} - \text{S} - \text{S} - \text{Keratin} + \text{R} - \text{SH} \leftrightarrow \text{Keratin} - \text{SH} + \text{R} - \text{S} - \text{S} - \text{R}$$

Examples include N-acetylcysteine, thioglycolic acid, mercaptoethanol and N-(2-mercaptoethyl) glycine (MPG). An increase in the surface roughness and porosity of both human nail plate and bovine hoof membrane treated with N-acetylcysteine has
been reported (48). The significantly increased permeation of triamcinolone across the latter has been observed. It was later confirmed (51) that the perturbation effect of N-acetylcysteine induced a conformational change in nail protein and increased the electrical conductivity of the barrier. An enhanced flux of caffeine across the nail plate pre-treated with thioglycolic acid has also been reported (73). Importantly, infrared spectroscopy has recorded the disappearance of S-S bond stretching from porcine hoof treated with mercaptoethanol, substantiating the reducing effect of thiol compounds (66). Moreover, a significant and potentially irreversible effect was induced by MPG, and was further increased when co-applied with urea (71).

Sulphites may also reduce S-S bonds (71):

\[
\text{Keratin} - S - S - \text{Keratin} + SO_3^{2-} \leftrightarrow \text{Keratin} - S - SO_3 + \text{Keratin} - S^-
\]

However, their variable effects have been less studied than those of thiols. Although the improved penetration of 5,6-carboxyfluorescein in the presence of sodium sulphite has been reported (74), Malhotra and Zatz (71) did not observe any increase in the penetration of water with 10% sodium metabisulphite, either alone or in combination with 20% urea. It appears that the relatively weak reducing ability of sulphites means that a long treatment period is required to break sufficient S-S linkages and elicit an enhancement effect.

Keratinases

The use of keratolytic enzymes has also been proposed as an approach to undermine the nail barrier. Papain serves as a potential candidate. Indeed, pre-treatment for 1 day with this enzyme, followed by 20% salicylic acid for 10 days, promoted the nail permeation of imidazole antimycotics (72). The separation of the dorsal nail plate cells (Figure 1.15) when treated with keratinase has been observed (75). The enzyme appears to attack the intercellular matrix protein, which holds cells together, causing them to ‘lift off’ the nail plate. Despite the surface corrosion, the deeper nail layer remained unaltered, presumably due to the limited penetration of the large enzyme molecule (MW 33000) into the nail plate. However, the more superficial effect may be sufficient to enhance ungual drug permeation, an increased penetration of metformin hydrochloride (MW = 166) into the enzyme-treated hoof membrane having been reported (75).
Figure 1.15: SEM images of (a) the dorsal surface, and (b) a cross-sectional view of the nail plate after incubation with 10 mg/mL keratinase. Reproduced with kind permission from (75).

1.5.3 Iontophoretic delivery

Recently, transungual iontophoresis, the application of a small electrical current to provoke the transport of (usually) charged molecules into the membrane has shown promise in enhancing drug permeation across the nail. While electrorepulsion is the major driving force for charged permeants, electroosmosis is an important flux enhancing mechanism for neutral molecules (76). The significantly higher in vitro permeation of ciclopirox olamine (77) and terbinafine hydrochloride (78), respectively, across human nail plate with iontophoresis has been demonstrated and the feasibility and tolerability of the approach has been confirmed in vivo (79, 80). Although iontophoresis caused transonychial water loss (TOWL), a marker for nail barrier function, to increase, compared with the simply hydrated control; the effect was transient, returning to baseline within 1 hour post-application. The efficacy and safety of topical treatment with terbinafine using an iontophoretic patch have also been demonstrated in a clinical study (80).

1.6 Polymeric nanoparticles

In this thesis, a novel approach of using polymeric nanoparticles (NPs) in topical nail delivery is investigated. Polymeric nanoparticle-based drug delivery systems have been widely studied. These are colloidal particles of less than 1 µm in diameter which have shown good stability, high loading capacity, the ability to incorporate both hydrophilic and lipophilic drugs and the potential to deliver an ‘active’ for sustained periods via different routes of administrations. NPs can be produced from natural or synthetic polymers, with the latter offering better purity (81). Commonly used polymers in nanoparticulate systems are poly(ε-caprolactone) (PCL) (82, 83), poly(lactic acid) (PLA)
(84, 85), poly(lactic-co-glycolic acid) (PLGA) (86, 87), poly(butyleyanoacrylates) (88), poly(methyl methacrylate) (89, 90), and polystyrene (91). Polymeric NPs typically fall into two categories, namely nanospheres and nanocapsules (Figure 1.16). The former have a solid mass and drug may be absorbed at the surface or entrapped within the polymer matrix. Nanocapsules are vesicular systems and the encapsulated active is either confined within the liquid core or adhered to the surrounding solid polymer shell (92). Various preparation techniques have been described and are generally classified into (a) in situ polymerization or (b) precipitation of pre-formed polymer. The commonly used methods are summarized in Table 1.6 (92-94):

Figure 1.16: Schematic representation of nanosphere and nanocapsule.
Table 1.6: Polymeric nanoparticle preparation methods.

(a) *In situ* polymerization

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion polymerization</td>
<td>Monomer is dispersed in water using an emulsifying agent to form monomer-containing micelles. An initiator (typically a free radical) diffuses into the micelle and polymerizes the monomer, leading to the formation of nanospheres.</td>
</tr>
<tr>
<td>Interfacial polymerization</td>
<td>Polymerization occurs between two reactive monomers at the interface of two immiscible solvents. This method is commonly used to produce core-shell-structured nanocapsules.</td>
</tr>
</tbody>
</table>

(b) Precipitation of pre-formed polymers (followed by solvent evaporation)

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoprecipitation</td>
<td>It is also known as the solvent displacement method. Water is usually used as a non-solvent into which is added a water-miscible solvent containing dissolved polymer. Upon adding the polymer solution into the aqueous solution containing surfactants, the solvent diffuses rapidly into water. This leads to the instantaneous formation of nanoparticles. Finally, the polymer solvent is removed by evaporation or centrifugation.</td>
</tr>
<tr>
<td>Emulsion solvent evaporation</td>
<td>Polymer is dissolved in a water immiscible organic solvent, which is then emulsified in an aqueous phase containing stabilizer. High-speed homogenization or ultra-sonication is generally employed to induce the formation of nanodroplets during the emulsification process. Subsequent removal of the organic solvent leads to the formation of nanoparticles, typically nanospheres.</td>
</tr>
<tr>
<td>Salting out</td>
<td>Polymer is dissolved in a water-miscible organic solvent, which is subsequently emulsified into an aqueous phase containing a stabilizer and a high concentration of salt or sucrose. The resulting emulsion is then diluted with a sufficient amount of water. This reduces the salt concentration in the aqueous phase and induces the diffusion of organic solvent out of the polymer droplets, leading to polymer precipitation in the form of spherical nanoparticles.</td>
</tr>
</tbody>
</table>

1.6.1 Polymeric nanoparticle-based formulations for topical application

Drug encapsulation using polymeric NPs offers an approach to modify the intrinsic physicochemical properties of the incorporated molecule and facilitate its percutaneous delivery. It appears that NPs not only increase a drug’s availability within the skin but also enhance and sustain its penetration into the barrier (95). NPs composed of different polymeric materials have been investigated for their potential as reservoir systems for topical and/or transdermal drug administration. Some examples are discussed below.

In cosmetic formulations, the incorporation of antioxidants, retinyl palmitate (96) and vitamin K₁ (97) in nanocapsules of PLA and PCL, respectively, increased the uptake of both compounds into the skin.
The application of polymeric NPs on sunscreen formulations has also been investigated. A nanocapsule gel-preparation of octyl methoxycinnamate (OMC), a highly lipophilic UV filter, provided better protection against UVB-induced erythema than a conventional gel. These results were attributed to the accumulation of NPs at the superficial layer, which efficiently cover the skin surface and improve the sunscreen’s ability to protect against UV damage (98). Subsequently, it was shown that PCL NPs produced a 3.4-fold higher level of OMC within the stratum corneum than that from the non-particulate formulation (82). Similar results have also been reported on the encapsulation of another sunscreen, benzophenone-3, into fatty-acid conjugated polyvinylalcohol (PVA) NPs (99).

Hair follicles represent an important route of uptake for NPs (89, 100, 101). The increased in vitro and in vivo penetration of minoxidil, a drug administered topically for the treatment of hair loss, into guinea pig skin with poly(ε-caprolactone)-block-poly(ethylene glycol) NPs has been demonstrated (102). Hair growing activities in mice in vivo were also enhanced by the use of PLGA nanospheres (86). Furthermore, a significantly increased permeation of flufenamic acid, an anti-inflammatory drug, into the deeper skin layer from PLGA (87) and propyl starch (103) NPs has been reported.

Finally, the antiseptic effect of chlorhexidine-loaded PCL NPs has been evaluated (83). While the NPs were found to locate at the skin surface, a higher level of chlorhexidine was delivered to the stratum corneum from the NPs than an aqueous solution. A sustained antimicrobial activity was also achieved using the particles. It was suggested that the NPs provided a direct, prolonged contact for the sustained delivery of chlorhexidine into the skin.

1.6.2 Novel use of nanoparticles in the treatment of nail diseases

The use of NPs in drug formulations for the treatment of nail diseases has received limited attention. Two examples are perhaps worth comment although, in both cases, it appears that drug delivery is eventually achieved indirectly via the skin (as opposed to across the nail plate itself). NanoBio Corp. (Ann Arbor, Michigan, USA) has developed an oil-in-water nanoemulsion as a topical antifungal treatment. It contains oil droplets (~180 nm diameter) stabilized by a quaternary ammonium surfactant, cetylpyridinium, which may also have an anti-infective effect. Broad-spectrum fungicidal activity has been demonstrated and fluorescence microscopy suggests that the active is taken up
appendageally and then accesses the infected nail by lateral diffusion from the epidermis \((104)\). Although Phase II clinical trials have been completed, no data have yet been published. The formulation of terbinafine into the nanoemulsion is a further objective \((105)\). Celtic Pharma (London, U.K.) has introduced a transfersome-based spray formulation (also with terbinafine as the active) for the treatment of onychomycosis. Transfersomes are ultra-deformable lipid vesicles \((106)\). High mycological cure rates and good clinical effect on treated nails have been claimed \((107)\). The transfersomes are believed to facilitate the percutaneous uptake of terbinafine and that lateral diffusion must again take place for the drug to reach the infected nail bed; however, the evidence for the proposed mechanism may be described as circumstantial at best.

The potential for using nanoparticles in some way as components of topical nail formulations is essentially unexplored, therefore, and a real need exists for more credible and objective research in the area to develop a sound rationale for the strategy and to provide an underpinning scientific understanding upon which a development and optimization programme might be based. This goal is one focus of the research described in this thesis.

1.7 Visualization of transungual permeation

*In vitro* nail permeation studies typically quantify the amount of active permeating from the dorsal side to the ventral, having crossed the entire nail plate. However, very little is known about the mechanism of permeation. It is established that water, a small polar molecule, penetrates the nail rapidly, but most of the drugs used to treat nail diseases are relatively large (compared to water) and rather lipophilic; i.e., not that compatible with the hydrophilic nature of the nail. Although in principle pathways of transungual permeation include transcellular (transport through the nail plate cells) and/or paracellular (transport via the intercellular spaces), there is a paucity of information and evidence that speaks to the role of each one for different compounds of interest. Furthermore, the establishment of a drug concentration gradient across the nail has not been well studied, and the depth to which penetration has occurred as a function of time remains poorly defined. Drug distribution across different layers (dorsal, intermediate or ventral) of the nail plate has only been determined by destructive methods. For example, a treated nail is milled to an approximate depth \((62, 108)\) or sectioned using a microtome \((109)\), and the concentration of drug within the harvested material can then
be assayed for the active. Obviously, these procedures provide only a crude measure of the drug level to a particular depth at a single time. To assess depth profiles as a function of time therefore requires multiple, different samples and introduces significant variability into the measurements. Because of these limitations, there is motivation and logic to attempt to visualize nail permeation using non-destructive imaging techniques. In this thesis, therefore, laser scanning confocal microscopy (LSCM), two-photon fluorescence (TPF) imaging, and a label-free technique, namely stimulated Raman scattering (SRS) microscopy, have been examined for this purpose.

1.7.1 LSCM and TPF imaging

LSCM (Figure 1.17), invented in 1978, permits direct imaging of a fluorescent target at multiple depths without mechanical sectioning. In conventional (wide field) microscopy, the entire sample is illuminated uniformly and simultaneously. As the emitted light from the focal plane is detected at the same time as the out-of-focus light from the areas above and below it, blurred images with poor resolution are generated. In contrast, illumination in confocal microscopy is not simultaneous but sequential, and is finely focussed on the specimen as a spot. There is also a spatial filter, an aperture or pinhole, which blocks all fluorescence other than that coming from the in-focus plane of interest. Optical sectioning is achieved by scanning x-y planes (i.e., parallel to the plane of the sample surface) successively in the z-direction, and thereby allowing a three-dimensional view of the sample to be obtained (110).

While LSCM is a single-photon excitation process, TPF (Figure 1.17) utilizes two photons, the combined energy of which is sufficient to excite the fluorophore. Using a pulsed laser, a high peak power delivers the necessary photon density to generate significant excitation at the focal plane and two photons are absorbed by the fluorophore simultaneously. With the strong focussing capability of the microscope objective and the quadratic dependence of the probability of two-photon absorption on the excitation power, high lateral and axial resolution, as well as optical sectioning, are therefore achieved (and excitation outside the focal volume is negligible). The longer excitation wavelength also results in less scattering, thereby improving imaging penetration depth. Because TPF restricts the excitation volume to the focal plane (unlike LSCM), photo-bleaching and photo-damage of the sample are minimized (111).
**Application of LSCM and TPF in skin and nail research**

LSCM and TPF have made important contributions to skin research, providing morphological and mechanistic information, particularly in terms of the preferred penetration pathways following the use of different enhancement strategies (for example, the visualization of vesicle-mediated drug transport). Due to the irregular nature of the skin surface, the standard approach of tissue fixation and mechanical sectioning is not well suited to the localization of topically-applied nanoparticles, the deposition of which into a skin fold or invagination is sometimes misinterpreted as evidence of deep penetration into the tissue. LSCM and TPF imaging avoid this potential artefact optically sectioning of skin samples that have undergone essentially no preparation steps whatsoever (112). Campbell et al. (113), using LSCM, adopted an objective, statistical strategy, taking into account the confounding effect of autofluorescence and inter/intra-sample variability, to study the distribution of fluorescent polystyrene nanoparticles (20-200 nm) in porcine skin. A lack of particle penetration beyond the superficial layer (2-3 µm) of the stratum corneum but localization in and around the hair follicles was visually demonstrated. Spatial
distribution analysis of the particle fluorescent signal confirmed the absence of time-
dependent penetration, even when the skin was partially damaged by tape stripping.
Using TPF, Stracke et al. (114) traced the time-dependent migration of fluorescein
(FA)-labelled PLGA nanoparticles (300 nm) encapsulating Texas Red (a model
penetrant) in excised human skin. While the particles remained on the surface of the
stratum corneum and deposited within the skin dermatoglyphs, the release of Texas Red
and its penetration into the skin was clearly visible (Figure 1.18).

![Figure 1.18](image)

Figure 1.18: (a) Planar image at a depth of ~32 µm, and (b) re-constructed orthogonal
images, of excised human skin exposed to fluorescein-labelled nanoparticles (green)
encapsulating Texas Red (orange). In the top-left image of (a), the grey scale shows
keratin autofluorescence and that from the nanoparticles; the top-right and lower-left
panels show the nanoparticles lodged in a skin ‘crevasse’ and the Texas Red released
into the skin, respectively; the lower-right panel is an overlay of the previous two
images. In (b), the sequestered green nanoparticles are seen in skin invaginations, while
the liberated Texas Red diffuses across the skin. Reproduced with kind permission from
(114).

Use of fluorescence microscopy in nail research is much more limited. For example,
LSCM has been used to study the morphological features (115) and to diagnose
onychomycosis (116) in vivo. Dutet and Delgado-Charro (117), on the other hand,
imaged the passive and iontophoretically-driven penetration of sodium fluorescein (SF)
and Nile blue chloride (NBC), into the nail in vitro. From the mechanically sectioned
transverse images and the three-dimensional reconstructed orthogonal views, the
slightly deeper penetration of the fluorophores into the nail with iontophoresis was
observed (Figure 1.19).
Figure 1.19: Representative confocal images showing the penetration of SF (green) and NBC (blue) passively and with an iontophoretic current of either 0.1 mA or 0.4 mA. Arrows indicate the nail surface to which fluorophores were applied. Panels A1 and B1 are images from mechanically sectioned samples allowing direct measurement of the penetration depth (short red line) and the total nail thickness (long red line). Panels A2 and B2 are orthogonal images generated from reconstruction of successive x-y planar views. The depth of penetration is indicated. Reproduced with kind permission from (117).

Limitations of LSCM and TPF
Despite their attractive ability for optical sectioning, LSCM and TPF are limited by the need for fluorescent markers and the laser frequency range(s) available. Typically, fluorophores are at best only approximate molecular surrogates for the real drug of interest and rarely have representative permeability characteristics. Furthermore, although autofluorescence is sometimes beneficial for revealing structural information about the sample, it can also interfere with, or even overwhelm, the emission from the target fluorophore, especially when the latter is present in small amounts. It follows that a preferred imaging tool would be label-free while maintaining high-resolution and z-profiling capability.
1.7.2 SRS microscopy

Raman microscopy provides an alternative technique that enables label-free imaging (118-120). It is based on the Raman scattering effect (121). When light interacts with a molecule, two types of scattering events occur: Rayleigh (elastic) and Raman (inelastic). Rayleigh scattering is the predominant mode, in which no energy is transferred from the photon to the molecule. Therefore, the scattered photon has the same energy (frequency and wavelength) as the incident photon. However, a small fraction (1 in $10^7$) of light undergoes Raman scattering. Here, the scattered photon is generated at the Stokes or anti-Stokes frequencies, having lower or higher in energy than the incident photon, respectively. Stokes transition occurs when the molecule is excited from a ground to a ‘virtual’ energy state and returns to its vibrational mode; anti-Stokes scattering occurs when, at the time of interaction, the molecule is already in excited vibrational state and the excess energy is released as it returns to its ground state. Figure 1.20 shows a simplified energy diagram that illustrates these concepts. The amount of energy required to excite a molecular vibration depends on the masses of atoms involved in the vibration, the type of chemical bonds between these atoms, and the interactions between the molecule and its environment. Consequently, each molecule has independent Raman-active vibrational modes, and, therefore, Raman spectra are highly molecule-specific.

![Energy levels and transitions of Rayleigh (elastic), Stokes Raman and anti-Stokes Raman (inelastic) scattering.](image)

Spontaneous Raman scattering (Figure 1.21a) uses one laser beam to illuminate the sample and the Raman signal, which depends linearly on the incident light intensity, is typically very weak. The long signal acquisition times also limit the use of spontaneous Raman for imaging.
Coherent Raman scattering, including stimulated Raman scattering (SRS) and coherent anti-Stokes Raman scattering (CARS), shown in Figure 1.21b and Figure 1.21c, respectively, improves Raman signal by means of non-linear excitation and allows imaging at a faster speed. In this case, two laser beams, pump and Stokes, with a frequency difference matches the molecular vibrational frequency, irradiate the sample. For CARS, a new signal at a different (anti-Stokes) frequency to the incident laser beams is generated and therefore, detection is relatively straightforward, using a filter and photomultiplier tube.

For SRS, signal is detected as a change in intensity between the pump and Stokes beams. When the energy difference between these two beams matching the energy of the molecular vibration, the excited state of the functional group of the molecule is populated. To achieve this transition, energy is provided by the incident lasers and due to the laws of conservation of energy, a photon from the pump beam is annihilated and a photon is created for the Stokes beam. In other words, the intensity of the pump beam decreases (stimulated Raman loss, SRL), while the intensity of the Stokes beam increases (stimulated Raman gain, SRG). These intensity changes ($\Delta I$) can be probed as chemical-specific contrast.

At biocompatible laser powers, $\Delta I$ are, however, very small relative to the intensity of the excitation beams ($I$) ($\Delta I/I < 10^{-4}$) and are typically buried within the laser noise, which exists primarily at low frequencies. To circumvent this problem, SRS microscopy utilizes a high-frequency modulation transfer scheme (Figure 1.22a). In this approach, one excitation beam is amplitude-modulated at a high frequency. The two beams are then aligned and interact with the sample, after which, the originally un-modulated beam is detected at the reference frequency using a photodiode and lock-in amplifier (Figure 1.22b). Since SRS only occurs when both laser beams are present, amplitude modulation is transferred from one beam to another and can be sensitively detected. The measured SRL or SRG provides each pixel of the image. Three-dimensional optical sectioning can be achieved using a confocal microscope.
Figure 1.21: Energy level diagrams for (a) spontaneous Raman, (b) CARS and (c) SRS. $\Omega$ = molecular vibrational frequency.

Figure 1.22: (a) Modulation behaviours of the input and output pulse trains. The resulting SRL in the pump beam is detected by the transferred amplitude modulation. (b) Diagram of a SRL microscope set-up. The pump beam and the high-frequency modulated Stokes beam are focused onto a common focal spot on the sample. After interaction with the sample, SRL of the pump beam is collected and detected by a photodiode and a lock-in amplifier. Redrawn based on (122).

**Advantages of SRS over CARS**

Although both SRS and CARS offer label-free imaging, the former is a more suitable technique for monitoring drug delivery. Firstly, SRS offers excellent vibrational contrast and chemical selectivity because no signal is detected when the frequency difference between the pump and Stokes beams does not match with a vibrational frequency of the sample. In contrast, the sensitivity of CARS is limited by its non-resonant background. Moreover, SRS allows more straightforward image interpretation and/or quantitative analysis because the signal output (Figure 1.23) is identical to the spontaneous Raman spectra and has linear concentration dependence, whereas CARS generates distorted vibrational spectra and the concentration dependence is quadratic (122).
Figure 1.23: (a) Raman peak of retinol by spontaneous Raman scattering, SRS and CARS. A peak shift and the non-resonant background are seen in the CARS spectrum. SRS signal output is identical to that of the spontaneous Raman scattering. (b) Linear dependence of SRS signals on the concentration of retinol. Reproduced with kind permission from (122).

Applications of SRS in skin imaging

The high-resolution, three-dimensional imaging capability of SRS has been demonstrated in a study of the physiological structures within the skin (119). Targeting the CH$_2$ stretching vibration (2845 cm$^{-1}$), the lipid rich regions at various depths into excised mouse skin, including the stratum corneum, the sebaceous gland and the subcutaneous fat layer were clearly visible (Figure 1.24). The distribution of dimethyl sulphoxide (DMSO) and retinoic acid (RA) into the skin was also traced by mapping the S=O (670 cm$^{-1}$) and the conjugated C=C bond stretching (1570 cm$^{-1}$), respectively (Figure 1.25) (119). While the hydrophilic DMSO partitioned predominately at the protein phase, the hydrophobic RA permeated exclusively through the lipid-rich intercellular spaces. Recently, the Xie’s group (123) has also demonstrated the use of video rate SRS imaging to observe the penetration of drug and excipients into the skin of living mice and human.

Figure 1.24: SRS CH$_2$ (2845 cm$^{-1}$) images of mouse ear skin at the indicated depths of (a) 4 µm (stratum corneum), (b) 42 µm (sebaceous gland) and (c) 105 µm (subcutaneous fat layer). Reproduced with kind permission from (119).
Figure 1.25: Top view of (a) DMSO and (b) RA in the stratum corneum, imaged with SRS contrast of 670 cm\(^{-1}\) (S=O) and 1570 cm\(^{-1}\) (conjugated C=C), respectively. (c) Simultaneous two-colour image of DMSO (green) and CH\(_2\) lipid (red) in the subcutaneous fat layer at a depth of \(\sim 65\) µm. Reproduced with kind permission from (119).

Saar et al. (124) employed SRS in a study of dermatopharmacokinetics by tracking the permeation of ketoprofen in a vehicle of deuterated propylene glycol (PG-d\(_8\)) into murine skin (\textit{ex vivo}). The penetration of drug and solvent were independently monitored by tuning the laser to the relevant wavelength (1599 cm\(^{-1}\) and 2120 cm\(^{-1}\), respectively) while the skin itself was captured by tuning to the CH\(_2\) stretching frequency (2845 cm\(^{-1}\)). A time-dependent penetration of ketoprofen and PG-d\(_8\) into the skin was clearly visible. The chemical disposition profiles were also determined using the signal intensities (Figure 1.26). It was found that the signals arising from both the drug and the vehicle within the intercellular lipid regions of the corneocytes increased steadily over time. A transfollicular route of uptake with limited capacity was also identified. The signal of PG-d\(_8\) from the hair shaft reached steady state rapidly before the first measurement (at 26 minutes post-application).

Figure 1.26: (a) SRS image showing the contrast of PG-d\(_8\) in the areas surrounding the corneocytes (red box) and at the hair shaft (green box). (b) Temporal profiles of PG-d\(_8\) at a depth of 6 µm into the skin. The red and green traces correspond to the areas enclosed by the corresponding boxes in (a), representing the intercellular and transfollicular pathways of penetration, respectively. Reproduced with kind permission from (124).
SRS also provides visible information on the phenomenon of metamorphosis of topically administered drug formulations. Figure 1.27a and Figure 1.27b show the images of (deuterated) ibuprofen crystals which were found on the surface of mouse skin (124) and porcine skin (125), respectively, within 30 minutes post application of the drug at ~90% saturation in PG. This offers a direct, visible proof of one reason for poor drug bioavailability of topical formulations that the penetration of excipients into the skin is faster than the active, leading to drug crystallization in situ.

Figure 1.27: SRS images showing the crystallization of ibuprofen-d₃, in PG on the surface of (a) mouse and (b) porcine skin within 30 minutes post-application. Images were obtained at 2120 cm⁻¹. Reproduced with kind permissions from (124) and (125), respectively.

1.8 Thesis outline

This project aims to characterize, and obtain mechanistic insight, into topical nail delivery, primarily using different imaging techniques. The main target of this work is to explore the potential of polymeric nano- or sub-micron particles to deliver drug into and across the nail by tracking their behaviour following topical application. The effect of other formulation approaches, including nail perturbation using microneedles, chemical treatment with penetration enhancers, and the use of a thermoresponsive nail lacquer, are also evaluated.

Chapter 2 demonstrates the novel application of stimulated Raman scattering (SRS) microscopy for direct visualization of the time course and depth of solvent penetration into the nail plate. The kinetics of solvent uptake is also characterized using the target signal intensities.
In Chapter 3, topical nail delivery using poly-methacrylate nanoparticles is examined. The distribution of the nanoparticles and of the model active is imaged using laser scanning confocal microscopy (LSCM).

Sustained drug delivery from poly(ε-caprolactone) nanoparticles into microneedle-porated nails is investigated in Chapters 4 and 5. The distribution of a fluorescent model active within the barrier following topical application of nanoparticles is first imaged with LSCM; subsequently, the localization both of fluorescently-labelled nanoparticles and of a lipophilic ‘active’ on and within the nail are tracked using two photon fluorescence (TPF) and SRS microscopies in combination.

Chapter 6 investigates the delivery of an antifungal drug into and across the nail from sub-micron, oil-filled poly(ε-caprolactone) particles. The effects on drug permeation of nail hydration, and pre-treatment with a chemical enhancer and microneedle-poration, are evaluated.

Finally, in Chapter 7, the development of an aqueous-based, *in situ* thermogelling formulation for topical nail treatment is described. The penetration of hydrophilic and lipophilic compounds from the gel into the nail is visualized with LSCM. The gelling behaviour is also evaluated *in vivo*.
1.9 References


74. Ng YJ, Mohorcic M, Torkar A, Friedrich J, Kristl J, Murdan S. Sodium sulphite – a potential onycheal enhancer to increase the topical drug delivery to the nail. AAPS J. 2007; 9(S2): T3181
Chapter 1


Chapter 1


Chapter 2: Assessment of solvent diffusion into the nail plate by stimulated Raman scattering (SRS) microscopy

Overview

Purpose: To visualize and characterize the real-time penetration of topically applied solvents into human nail plate using stimulated Raman scattering (SRS) microscopy.

Methods: Deuterated water (D$_2$O), propylene glycol (PG-d$_8$) and dimethyl sulphoxide (DMSO-d$_6$) were separately applied to the dorsal surface of the nail plate. SRS microscopy was used to image D$_2$O, PG-d$_8$/DMSO-d$_6$ and the nail through the O-D, -CD$_2$ and -CH$_2$ bond stretching Raman signals, respectively. Signal intensities obtained were measured as functions of depth into the nail and of time.

Results: The time-dependent penetration of the solvents into the nail was clearly observed with the diffusion of D$_2$O being more than an order faster than that of PG-d$_8$ and DMSO-d$_6$. Semi-quantitative analysis of the permeation profiles strongly suggested that solvent diffusion was concentration-dependent and non-Fickian. It appeared that the uptake of solvent progressively undermined the integrity of the nail.

Conclusions: This novel application of SRS has permitted direct visualization and semi-quantititation of solvent penetration into the nail plate. The kinetics of uptake of the three chemicals studied demonstrated that each altered its own diffusion in the nail in a concentration-dependent fashion. The scale of the unexpected behaviour observed may prove beneficial in the design and optimization of topical nail formulations.
Chapter 2

2.1 Introduction

The effective treatment of nail disease requires efficient drug delivery into and through the barrier. However, the tightly woven keratin network of the nail plate means that poor drug uptake following topical administration is common. Despite considerable effort to improve formulations and to enhance drug delivery to the nail, progress has been slow at best. In general, the approaches adopted have failed to understand the complex interplay between drug, formulation components (including solvents) and the nail. For example, although it is quite clear that drug uptake from typical ‘lacquer’ formulations (comprising the active, a film-forming polymer and a volatile organic solvent) is intimately linked to the disposition of the solvent, and effectively stops once the solvent has gone, there has been little effort to characterize the transport of these key vehicle components into and across the nail. Only the diffusion of water has received attention, its overall time-dependent uptake having been measured by various techniques (1-3); otherwise, apart from some information on the concentration-depth profiles of water and dimethyl sulphoxide (DMSO) in the very superficial, outermost 20 µm of the nail, there are essentially no time and position-dependent data on the movement of chemicals into the nail.

Stimulated Raman scattering (SRS) microscopy is a label-free imaging technique that offers a solution to this challenge. This method has been applied in a range of biomedical and pharmaceutical studies involving, for example, visualization in living cell (4), characterization of cortical vasculature morphology (5), imaging the constituents of solid, oral dosage forms (6) and tracking the dermatopharmacokinetics of drugs and excipients in mammalian skin (7-9). In this paper, the first application of SRS microscopy to trace and visualize the diffusion of three pharmaceutically relevant solvents, water, propylene glycol (PG) and DMSO, as a function of depth and in real time, in human nail is presented. The use of deuterated solvents provides unique Raman-active molecular vibrations that are easily distinguished spectroscopically from those originating in the nail, resulting in excellent image contrast without significant modification of molecular weight of the solvents which is a key parameter governing transport kinetics across the barrier (10). Because of the linear relationship between the SRS signal and the concentration of the chemical, the spectroscopic signature of which is being monitored, a semi-quantitative analysis of solvent diffusion across the nail is possible and offers heretofore-unknown insight into the transport process.
2.2 Materials and methods

2.2.1 Sample preparation
Deuterated water (D$_2$O), PG-d$_8$ and DMSO-d$_6$ were purchased from Sigma-Aldrich Co., Ltd. (Gillingham, U.K.). Human nail clippings were obtained from healthy volunteers and stored at -20°C until use. The University of Bath Research Ethics Approval Committee for Health (REACH; EP 11/12 115) granted ethical approval for nail sampling, and all individuals donating nails gave informed consent (Forms in Appendix 1). Each nail sample was carefully cleaned with deionized water and dried with absorbent tissue before each experiment. Prior to imaging, 5 µL of neat solvent was applied over the nail (~16 mm$^2$ in area) which was then sandwiched between two glass coverslips within a Parafilm® frame, which acted as a spacer. The coverslips were sealed by melting the Parafilm® and further secured using double-sided tape. This ensured that the sample was tightly sandwiched to minimize solvent evaporation and sample dehydration during the time-lapse experiments.

2.2.2 Raman spectroscopy
To identify suitable vibrational bond resonances for each solvent prior to SRS imaging, their Raman spectra, and that of a nail clipping sample, were acquired using a Raman microscope (Renishaw RM1000, Renishaw plc, Wotton-under-Edge, U.K.) and Renishaw v1.2 WIRE software. A 1200-line/mm grating providing spectral resolution of 1 cm$^{-1}$ was used with a diode laser operating at 785 nm. The Raman band (520 cm$^{-1}$) of a silicon wafer was used for calibration. The Vancouver Raman Algorithm (BC Cancer Agency & University of British Columbia, Canada) was employed for automated fluorescence background removal from the Raman spectra.

2.2.3 SRS imaging
The SRS microscope consisted of a picosecond laser system and a modified commercial inverted laser-scanning microscope with a confocal laser scanner (FV300/IX171, Olympus UK Ltd, UK). Synchronized, dual-wavelength picosecond excitation was provided by an optical parametric oscillator (OPO) (Levante Emerald, APE, Berlin) which was synchronously pumped at 532 nm by a frequency-doubled Nd:Vanadium laser (picoTRAIN, High-Q GmB), delivering a 7 ps pulse train at a 76 mHz repetition rate. The OPO consisted of a temperature-tuned, non-critically phase matched Lithium Triborate (LBO) crystal, which allows the OPO signal (employed as the pump beam) to
be continuously tuned from 690 to 980 nm by adjusting the LBO temperature and an inter-cavity Lyot filter. A Si PIN photodiode was used to record the intensity variations of the OPO signal. The pump-laser fundamental (1064 nm) was also available as a separate output and was used as the Stokes beam, which was amplitude modulated at 1.7 MHz with an acousto-optic modulator (3080-197 Crystal Technologies, West Chester, PA, USA).

The pump beam and the modulated Stokes beam were spatially overlapped using a dichroic mirror (1064 DCRB, Chroma Technology Corp, Bellows Falls, USA) and temporally overlapped using a delay stage. The collinear beams were directed into the microscope and focussed onto the sample using a 20X 0.75 NA air objective (UPlanSApo, Olympus) and scanned in two dimensions using a pair of galvanometer mirrors. The resulting SRL in the pump beam was collected in the forward direction via a 1.0 NA condenser lens (LUMFI, Olympus) and detected by a large area photodiode (FDS1010, Thorlabs, New Jersey, USA). A band-pass filter (850/90 nm, Chroma) was mounted in front of the detector to block the modulated 1064 nm beam. Finally, a lock-in amplifier (SR844, Stanford Research Systems, Sunnyvale, CA, USA) was used to detect the SRL signal with a time constant of 30-100 µs.

2.2.4 SEM analysis

Scanning electron microscopy (SEM) was used to investigate solvent effects on the integrity of the nail surface. A nail sample was cut into four pieces of approximately 4 mm² in area. Three were placed in sealed vials containing 1 mL of either water, PG or DMSO for 24 hours at room temperature; the fourth piece of nail served as an untreated control. Post-treatment, the nails were dried with tissue, mounted on the aluminium stubs using double-sided tape, and imaged by SEM (JEOL SEM6480LV, JEOL Ltd., Tokyo, Japan).

2.2.5 Data analysis

All acquired images were processed using ImageJ (U.S. National Institutes of Health, USA). Each data point was normalized against the OPO signal recorded on the PIN photodiode to correct for the laser intensity fluctuations. Images of different Raman shifts were presented using different colour schemes for ease of interpretation. Signal quantification of the image was performed using the ‘plot profile’ or ‘plot z-stack profile’ plugins, overlaid images were obtained using the ‘colour merge’ function and
3D images were produced using the ‘3D viewer’ function. Data fitting was performed using GraphPad Prism® version 5.00 (GraphPad Software, San Diego, CA, USA).

2.3 Results and discussion

2.3.1 Raman spectra

Raman spectra of a nail clipping and of the solvents investigated are shown in Figure 2.1. The unique Raman shifts, specifically -CH$_2$ bond stretching (2855 cm$^{-1}$) from the nail, -CD$_2$ stretching (2120 cm$^{-1}$) from PG-d$_8$ and DMSO-d$_6$, and O-D stretching (2500 cm$^{-1}$) from D$_2$O, are clearly identifiable. The off-resonance signal at 1802 cm$^{-1}$ serves as a suitable background at which neither the nail nor the solvents are Raman-active.

![Raman spectra](image)

Figure 2.1: Raman spectra of the nail and of the solvents studied.

2.3.2 Control images

Control images (Figure 2.2) from untreated nails were acquired using the -CH$_2$ stretching vibration; the nail surface was clearly observed. For the skin, this absorbance originates primarily from the lipids in the outermost layer, the stratum corneum (SC) (8). However, the lipid content of the nail is reported to be very low (0.1-1%) (11) (i.e., an order of magnitude or two less than that in the SC) and the -CH$_2$ signal more likely originates from keratin, the principal protein component present. No signal from untreated nails was observed either off-resonance or at the -CD$_2$ stretching frequency; the infrequent and small punctate ‘spots’ on the otherwise uniformly black images are due to two-photon absorption (TPA) (12) from residual dirt particles that were not removed when the nails were cleaned.
Figure 2.2: SRS images of an un-treated nail surface showing -CH₂ bond stretching (A), absence of -CD₂ signal (B) and the off resonance background (C). Scale bar = 50 µm.

2.3.3 Absorption of D₂O into the nail

To observe the absorption of D₂O into the nail plate, the SRS microscope was tuned to the O-D stretching vibration at 2500 cm⁻¹. After application of the solvent to the nail and positioning of the sample on the microscope stage, an x-z line scan (x = 353 µm) was performed at t = 10 minutes capturing every 1 µm in the z-direction into the nail. This scan (150 lines) was repeated on 9 further occasions, every 2.7 minutes (i.e., until t = 34.3 minutes post-application of the D₂O). Acquisition of each line scan required 1.07 seconds. Finally, the nail was imaged by re-tuning the SRS microscope to 2855 cm⁻¹ and an off-resonance background was recorded at 1802 cm⁻¹.

The results are presented in Figure 2.3 (and as a video in Supplementary data 2.1) which shows superimposed x-z orthogonal views of the O-D signals obtained from each scan as a function of time from 5 different regions (35 x 150 µm²) of the D₂O-treated nail. The SRS image from the -CH₂ contrast of the nail permits the surface to be clearly delineated; the off-resonance ‘image’ only reveals (as before) a very few bright points of light scattering due to residual particulate matter not removed by the cleaning process prior to starting the experiment.

The O-D signal recorded at each 2.7-minute interval is represented by a separate colour on the visible spectrum scale shown on Figure 2.3; that is, red corresponds to the measurement at t = 10 minutes, yellow to that at t = 12.7 minutes, and so on. These scans have then been superimposed (prepared using the ImageJ ‘transparent zero’ function), one upon the other, to generate (at each of the 5 different regions visualized) an image of D₂O diffusion into the nail. Within 35 minutes, it can be seen that the deuterated water had diffused approximately 100 µm into the sample. This relatively rapid uptake of water into the nail has been inferred from previous investigations (13, 14) but the transport process has never been visualized before in such a direct fashion. The results are consistent with the nail being characterized as a dense hydrogel
containing overlapping keratin fibres, which create small, tortuous, pore pathways that favour the permeability of small, hydrophilic molecules, such as water (15).

![Composite SRS x-z orthogonal view images of the penetration of D₂O into 5 regions of the nail as a function of time (the 5 panels on the right of the figure labelled O-D). The visible spectrum scale indicates the O-D signals recorded every 2.7 minutes, from t = 10 minutes (red) to t = 34.3 minutes (magenta) post-application. The SRS image from the keratin in nail is shown in the far left panel (labelled as ‘Nail’), while the background, off-resonance control is to the immediate right (labelled as ‘OR’). Scale bar = 20 µm.](image)

### 2.3.4 Penetration of topically-applied PG-d₈ and DMSO-d₆ into the nail as a function of time

To follow the penetration of PG-d₈ and DMSO-d₆ into the nail, SRS imaging at 2120 cm⁻¹ (the -CD₂ stretching vibration) was performed. At each time point, as the diffusion of these solvents was much slower than that of D₂O, images were also recorded for the nail (-CH₂ at 2855 cm⁻¹) and off-resonance (1802 cm⁻¹). In this case, x-y planar images (353 µm x 353 µm) were captured every 1 µm in the z-direction at each measurement time. The scan time for each frame was 18.4 seconds.

The time-courses of PG-d₈ and DMSO-d₆ absorption into the nail as a function of depth are shown in Figure 2.4. While the -CH₂ signal from the nail is relatively constant, the shorter time measurements (t ≤ 8 hours) reveal that uptake of PG and DMSO occurs only into the outer 15-20 µm of the nail. Only after about a day have the two solvents reached a depth of about 40-50 µm into the nail. Figure 2.5 illustrates an alternative, cross-sectional (x-z) view of PG and DMSO penetration that enables direct visualization of the solvents on and within the nail. Notably, and self-evidently, the rate of diffusion
of PG and DMSO is substantially less than that of D$_2$O (which had permeated 100 μm in only ~30 minutes), the molecular size of which is about one-quarter of that of the two other solvents: the molecular weights of water, PG and DMSO are 18.0, 76.1 and 78.1, respectively; the corresponding molar volumes are 18.0, 73.4 and 71.0 cm$^3$. The relatively poorer uptake of PG and DMSO into the nail has been reported (15, 16) and their penetration-enhancing abilities are less than clear-cut (16, 17). Rotating 3D composite images showing the progressive penetration of PG-d$_8$ and DMSO-d$_6$ into the nail are presented in Supplementary data 2.2a-d and 2.3a-e, respectively.
Figure 2.4: SRS x-y planar images of the penetration of (A) PG-d$_8$ (blue), and (B) DMSO-d$_6$ (green) into the nail (red) as a function of time and depth. OR shows the off-resonance background. Depths of images are indicated along the top while the experimental duration is indicated down the left-hand column. Scale bars = 50 µm.
2.3.5 SRS signal analysis and interpretation

To better interpret the results obtained, an attempt to more quantitatively analyse the SRS signals (specifically, the measured pixel intensities) from the three solvents was undertaken. To do so required a number of potentially confounding factors to be addressed, including: (a) definition of the nail surface, (b) fluctuations in SRS laser intensity, (c) movement of the sample (e.g., due to swelling), (d) artefacts caused by residual particulate matter on the nail, (e) variable off-resonance background signal, and (f) confirmation that no significant depletion of solvent at the nail surface had occurred by the end of the experiment.

Because of the natural curvature of the human nail, it is clear that a z-series of x-y planar images will not sample the same depth across the entire sample (see Figure 2.6) and it was therefore decided to define a virtual surface using the intensity of the -CH$_2$ signal from nail protein. To do so, 5 regions of the examined nail (35.3 x 35.3 µm$^2$ x-y planes for PG and DMSO, 20 µm sections for D$_2$O) were delimited avoiding those where either particulate matter or an air bubble in the solvent on the nail clearly interfered with the image. For each selected region, the nail surface was defined when the -CH$_2$ signal had reached 90% of its maximum value, thereby aligning the 5 surfaces...
on one horizontal line (Figure 2.6). This procedure also allowed for correction of any sample movement (typically no more than 1-2 µm) to be made as well. The small background off-resonance signal, when present, was subtracted from -CH₂ and O-D/-CD₂ signals in each image. The average pixel density of the solvent (as a function of depth into the nail) was then normalized by that at the defined surface (z = 0 µm), i.e., all signals from the solvents were then expressed as a fraction of that at the surface reflecting, in theory at least, a relative concentration profile of the compound across the nail. The solvent signals at the nail surface did not decay significantly over the time-course of the experiments confirming that no appreciable depletion had occurred and that an effectively infinite dose had been applied to the nail.

Figure 2.6: Schematic diagram illustrating the creation of a horizontal nail surface using the intensity of the -CH₂ signal from nail protein (red) when treated with a solvent (blue). This method was adopted to define z = 0 µm.

Nevertheless, despite these efforts to enable more quantitative analysis of the SRS imaging results, there remains the unresolved issue of signal attenuation with increasing depth into the nail due to light absorption and scattering (a problem ubiquitous to confocal imaging). For the moment, no satisfactory and validated approach has been developed to circumvent this limitation and it must be recognized that the signals emanating from deeper into the nail almost certainly reflect under-estimates of the actual amount of chemical present.

While fully appreciating this constraint, the average pixel intensity data extracted from the SRS images are presented graphically in Figure 2.7. The error bars (standard deviations) reflect the variability observed across the 5 sampled regions of the nail. This, at best, semi-quantitative representation of the results offers useful insight into solvent diffusion across the nail as a function of time and position. At shorter time points, the concentration profile decays monotonically as one would expect for non-steady state...
diffusion into a semi-infinite medium (18). However, clear deviation from this classic model is observed as the time of diffusion increases and distortion of the concentration profile of PG and DMSO at \( t \approx 1 \) day, for example, are clearly apparent with more solvent taken up than would be expected from simple Fickian diffusion (and recall that this is very likely less than the real amount for the reasons just discussed).

Figure 2.7: Normalized SRS signal versus nail depth profiles for D\(_2\)O, PG and DMSO as a function of time post-application (n = 5, mean + SD).

Further understanding of the SRS signal profiles is accessible by comparing their progressive deviation from the solution to Fick’s 2\(^{nd}\) law of diffusion for the semi-
infinite approximation, i.e., treating the nail as a homogenous plane sheet, with the boundary conditions: (i) the normalized solvent signal \((S/S_{z=0})\) at the nail surface \((z = 0)\) equals 1 at all times, \(t \geq 0\), (ii) at \(t = 0\), \(S/S_{z=0} = 0\) at \(z > 0\), and (iii) at \(t \geq 0\), \(S/S_{z=0} = 0\) at \(z = \infty\). In other words, first, during the course of the experiment, there is a constant source of solvent on the nail surface. Second, initially, there is no solvent in the nail. And, third, the nail can be considered infinitely thick such that no solvent diffuses all the way through during the observation period.

With these constraints, Fick’s 2nd law can be solved analytically (18) to yield the following expression for the evolution of the SRS profiles as a function of time and position:

\[
\frac{S}{S_{z=0}} = 1 - \text{erf}\left(\frac{z}{2(D_it)^{1/2}}\right) \quad (\text{Eq. 2.1})
\]

where \(D_i\) is the solvent diffusivity. As stated above, the shortest time SRS signal profiles for the three solvents reasonably followed this monotonic decay function and fitting these data to Eq. 2.1 permitted, therefore, an initial value of the solvents’ diffusivities to be deduced; the results are in Table 2.1. Notably, the estimated value for water is ~40-fold and 25-fold greater than those for PG and DMSO, respectively.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>(x10^{11} D_i(\text{cm}^2\text{s}^{-1}))</th>
<th>(r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{D}_2\text{O})</td>
<td>124 ± 60</td>
<td>0.82 – 0.99</td>
</tr>
<tr>
<td>PG-d(\delta)</td>
<td>3.07 ± 0.82</td>
<td>0.99 – 1.00</td>
</tr>
<tr>
<td>DMSO-d(\delta)</td>
<td>5.02 ± 0.76</td>
<td>0.97 – 0.99</td>
</tr>
</tbody>
</table>

To examine the extent to which the evolution of the SRS signal profiles for the solvents subsequently deviated from Eq. 2.1, the data in Figure 2.7 were plotted against the normalized parameter, \(z/(4Dt)^{1/2}\) (18). If the solvent diffusivity remains constant over time and position, the profiles as a function of time should be aligned and overlap. However, the outcome of this approach (Figure 2.8) shows that this is clearly not the case. For \(\text{D}_2\text{O}\), there is already a shift in the profile by 15 minutes, and the deviation becomes more pronounced as time increases further. The behaviour observed is consistent with the diffusivity of water increasing with its increasing uptake into the nail.
Rapid nail hydration when immersed in water is well known, and the resulting swelling/opening of the keratin structure is manifested by an uptake-dependent increase in diffusivity (2). Notably, the diffusion of water is substantially faster than that of the other two solvents examined. For PG, the normalized profiles overlapped during the first 7 hours of transport, but had clearly deviated by ~1 day, when an obvious ‘shoulder’ in the data had appeared (Figure 2.7 and Figure 2.8). The same behaviour was seen for DMSO although the deviation began sooner than that for PG. Like water, therefore, the results suggest strongly that the progressive uptake of the solvents impacts upon the nail structure and facilitates enhanced diffusion. The much swifter passage of water, relative to the other two solvents, despite only a 4-fold difference in molar volumes, implies that there must be a strong size-dependence to at least one transport pathway across the nail that is accessible to water but excludes PG and DMSO.

The non-ideal diffusion of the solvents across the nail can also be illustrated by comparing the areas under the SRS signal profiles in Figure 2.7 with those which would have been observed had the transport been purely Fickian with a constant value of $D_i$ (i.e., following Eq. 2.1). Figure 2.9 shows the outcome of this analysis and highlights the evolution and extent of the deviation for each of the three solvents. As described above, the impact of water on its own transport is substantial and almost immediate, while those of PG and DMSO are more subtle at first, and become more noticeable at longer times; the slightly enhanced onset of the effect of DMSO, relative to PG, can be discerned.
Figure 2.8: Normalized SRS signal of D₂O, PG and DMSO as a function of the composite variable, \(z/(4D_i t)^{1/2}\), using the value of \(D_i\) deduced from the earliest measurements for each solvent. For each solvent, the fits shown were obtained using the mean data (n = 5) and the LOWESS (fine) curve fit function in GraphPad Prism®.
Figure 2.9: Areas under the measured SRS signal versus depth profiles (AUC) in Figure 2.7 (red circles) compared with the corresponding values (black squares) predicted from Eq. 2.1 assuming constant values for the solvent diffusivities (mean + SD; n = 5).

SEM images of control nail samples and of those exposed to the three solvents for 24 hours are shown in Figure 2.10. The untreated nail surface is compact and relatively smooth, while solvent treatment appears to have loosened the structure and markedly increased surface roughness. It may be inferred, therefore, that the integrity of the outer nail has been compromised, at least to some extent, and this is consistent with recent research reporting an increase in surface porosity with hydration (19). Further precise details as to the molecular mechanism by which the uptake of a solvent facilitates its own diffusion across the nail cannot be deduced from the results obtained. Nonetheless, the SRS signal profiles, even with the important caveat that light absorption and scattering prevent any absolute quantification of the results, are fully consistent with the diffusivity of the solvents in the nail exhibiting concentration-dependent behaviour, a phenomenon that appears to be common (at least for water) across other keratinized tissues, such as hair and the stratum corneum (2, 20). It is worth noting that (because of the absorption/scattering limitation) that the effects observed and reported here are probably greater than those deduced from the results. Whether the solvent diffusional front proceeds uniformly and enhanced transport occurs in a similar fashion across the entire nail, or whether there are solvent ‘channels’ opened up at weak points in the barrier with increasing time of exposure to provide lower resistance pathways, remains to be seen.
Figure 2.10: SEM images of the dorsal nail surface, either untreated or exposed to water, PG or DMSO for 24 hours. The scale bars are 50 µm for the 4 panels on the left, and 10 µm for those on the right.

2.4 Conclusions

SRS microscopy has been successfully used to unambiguously visualize the uptake of water, propylene glycol (PG) and dimethyl sulphoxide (DMSO) into the human nail plate and to characterize the diffusion of these solvents across the tissue. Analysis of the SRS signal profiles revealed the much faster transport of water through the nail, relative to PG and DMSO. Furthermore, the results demonstrate that all three solvents progressively enhance their own diffusion through the nail: as more solvent is taken up, there is a distinct deviation from simple Fickian behaviour. This concentration-dependent diffusivity is consistent with scanning electron microscopy of the outer nail
surface that indicates each solvent’s ability to undermine the integrity of the tissue. Although the conclusions drawn can be only semi-quantitative in nature because the SRS signal profiles are attenuated (due to light absorption and scattering) as a function of depth into the nail, the interpretation of the effects observed is not altered by this limitation – indeed, if anything, the scale of the solvents’ deduced impact on the nail is almost certainly under-estimated.

The research described is significant as it offers insight into the practical challenge of drug formulation for the treatment of nail disease, an important unmet medical need. The substantial barrier properties of the nail mean that the rate and extent at which topically applied drugs can reach (e.g., fungal) targets in the nail plate are very limited. The results presented here show that optimization of delivery platforms to the nail must prolong and sustain exposure of the barrier to excipients, such as common solvents like water, PG and DMSO, that can facilitate both drug and their own transport. In this way, it is envisaged, it should be possible to develop new and improved formulations that significantly increase the availability of drugs at their site(s) of action in and/or beneath the nail.
2.5 References


Chapter 3: Topical application of nanoparticles and an associated lipophilic dye to the nail

Overview

Purpose: To investigate the use of poly (methyl methacrylate) (PMMA) and poly (methyl methacrylate-co-butyl methacrylate) P(MMA-co-BMA) nanoparticles (NPs) as drug reservoirs for sustained topical drug delivery into the human nail plate.

Methods: The NPs were labelled with fluorescein methacrylate (FMA) and loaded with a lipophilic fluorophore, Nile red (NR). The size and morphology of NPs were characterized by dynamic light scattering and transmission electron microscopy. The disposition of the NPs and NR following topical application to the nail in vitro was imaged by laser scanning confocal microscopy (LSCM). An in vitro skin permeation experiment was also performed for comparative purposes. Finally, the release of NR from the NP suspension into a lipophilic medium (isopropyl myristate, IPM) was determined.

Results: Spherical NPs were produced with diameters of ~20 nm. Confocal images showed clearly that both types of NPs located only at the nail surface after 7 days of application, with co-localization of fluorescein-labelled NPs and NR observed. Results from the skin experiments were similar. Consistent with these findings, negligible release of NR into IPM from the NPs occurred.

Conclusions: As anticipated, there was no evidence that even the very small NPs considered were able to penetrate into the nail. Instead, they remained at the surface where they retained NR, lipophilic model drug employed. Unfortunately, the affinity of NR for the poly-methacrylate-based NPs was such that no release into the nail (or, indeed, into a simple IPM receive medium) took place. Further work should therefore focus on modifying the NP polymer properties.
3.1 Introduction

Polymeric nanoparticles (NPs)-based drug delivery systems have attracted much discussion in the skin research field, especially with respect to their claimed ability to enhance the uptake/absorption of various compounds, including anti-inflammatory drugs (1, 2), antimicrobials (3, 4), hair growing promoters (5), sunscreens (6, 7), and even insulin (8). NPs offer the potential to mask unfavourable physicochemical properties of the encapsulated active (e.g. poor solubility), improve drug stability and enhance topical delivery (9). NPs have been shown to increase a drug’s residence time on the skin at the very least by accumulation on, or near, the surface, within the crevasses of the tissue, and via sequestration in and around hair follicles. An obvious advantage is that the particles can act as drug reservoirs, maintaining the incorporated active in a diffusible form which can be released and partition into the skin over a long period of time (3, 10-13). Conventional topical nail formulations have limited usefulness, due to both immobilization of drug once the volatile solvent in the formulation has evaporated, and the low diffusivity of drug across the compact keratinized structure of the nail plate. Exploitation of the reservoir potential of NPs has not been explored on the nail, however, and this study aimed, therefore, to examine whether sustained drug delivery into the nail might be possible from these polymeric structures.

In this study, NPs made of biocompatible methacrylate-based polymers were investigated. Methacrylate polymers have low toxicity and are commonly used as adhesive in the transdermal patch formulations (14). Here, poly (methyl methacrylate) (PMMA) and poly (methyl methacrylate-co-butyl methacrylate) P(MMA-co-BMA) NPs were synthesized using a microemulsion polymerization method, which has been widely adopted for the production of stable latex particles (diameter 10-100 nm) with narrow size distributions (15). The polymers were fluorescently-labelled by covalent attachment of fluorescein methacrylate (FMA) and particles were loaded with another fluorophore, Nile red (NR) (Figure 3.1), which acted as a lipophilic model drug for the purpose of this study. Laser scanning confocal microscopy (LSCM) was used to visualize the dual-labelled NPs following their topical application to the nail plate. A skin permeation study was also performed to compare the NP/NR disposition profiles between the two barriers.
Figure 3.1: Structure of NR. Molecular weight = 318 Da; average of predicted LogP (ALOGPS 2.1 program (16)) is 4.07 ± 0.26.

3.2 Materials and methods

3.2.1 Chemicals
Methyl methacrylate (MMA, 99%, contains ≤ 30 ppm monoethyl hydroquinone as inhibitors), butyl methacrylate (BMA, 99%, contains 10 ppm monomethyl ether hydroquinone as inhibitors), fluorescein methacrylate (FMA, 97% pure), Nile red (NR, analytical grade), sodium dodecyl sulphate (SDS, ≥98.5% GC), potassium persulphate (KPS), propylene glycol (PG) and isopropyl myristate (IPM) were purchased from Sigma-Aldrich Co. (Gillingham, UK).

3.2.2 Nail sample preparation
Human fingernail clippings (at least 8 mm in length) were obtained from healthy volunteers who gave informed consent. Ethical approval was granted by the Research Ethics Approval Committee for Health (REACH) (Forms in Appendix 1) of the University of Bath. Nails were maintained frozen (-20°C) until use. Prior to the experiments, the nail was soaked in deionized water for 30 minutes to restore some flexibility, and then mounted in a specially adapted Franz cell (PermeGear Inc., Bethlehem, PA, USA) with a diffusion area 0.2 cm².

3.2.3 Skin tissue
Porcine skin was obtained from a local slaughterhouse. After cleaning and trimming off coarse hairs, the tissue was dermatomed to a nominal thickness of ~750 µm. The skin was then frozen at -20°C until use. Before the permeation experiment, the skin was thawed and any (remaining) finer hairs were carefully trimmed as close as possible to the surface.
3.2.4 Nanoparticle preparation

Fluorescein-labelled NPs were prepared by free radical emulsion polymerization (15, 17) (Figure 3.2). Monomers, MMA and BMA, were first purified by passing through an inhibitor remover column (Sigma-Aldrich, Gillingham, UK). The aqueous phase was a 0.5% w/v solution of SDS, deoxygenated with nitrogen gas. NR (0.1% w/v) was added to the monomer mixture, which comprised FMA (0.06% w/v) in either MMA or 50/50 by weight MMA/BMA. This solution was added to the aqueous phase and an emulsion, containing 6.5% w/v of the organic phase, was formed with vigorous stirring. This emulsion was then heated to ~75°C and KPS (0.1% w/v), a free radical initiator dissolved in a small amount of water, was introduced to start the polymerization process. The reaction was allowed to proceed at constant temperature under nitrogen for 3 hours. The resulting NP suspension was then filtered (0.45µm nylon filters, Whatman, Maidstone, UK) to remove any large aggregates.

\[
\begin{align*}
\text{a} & \\
\text{MMA} + \text{FMA} & \xrightarrow{\text{KPS}} \text{PMMA-FMA} \\
\text{b} & \\
\text{BMA} + \text{MMA} + \text{FMA} & \xrightarrow{\text{KPS}} \text{P(MMA-co-BMA)-FMA}
\end{align*}
\]

Figure 3.2: Polymerization of (a) FMA and MMA, and (b) FMA, MMA and BMA.

3.2.5 Nanoparticle characterization

The average NP diameters were determined using dynamic light scattering (DLS) (ZetaSizer Nano S, Malvern, UK). The polydispersity index (from 0 to 1), which describes the particle size distribution, was also measured where a value of 0 indicates a monoparticle size distribution, while a value of 1 implies a large variation in particle sizes. Measurements were made in triplicate for each sample. The morphology of the NPs was studied by drop-casting the suspension onto a carbon coated copper grid and
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imaged using transmission electron microscopy (TEM) (JEOL JEM-2000, JEOL Ltd., Tokyo, Japan). Negative staining with uranyl acetate was employed to improve the contrast of the TEM images.

3.2.6 **In vitro nail and skin permeation studies**

Nail permeation experiments were performed using vertical Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) (Figure 3.3). The nail clipping was mounted in the nail adaptor with the dorsal surface facing the donor chamber, while the ventral side was fully hydrated by the pH 7.4 phosphate buffer solution (PBS). 300 µL of the NP suspension was introduced into the donor compartment, which was covered with Parafilm® to avoid excessive evaporation. The entire diffusion cell was then placed in an incubator at 32°C. Nails were also treated with deionized water and with a solution of NR in PG (90% saturation) (6) as negative and positive controls, respectively. After 7 days, the diffusion cell was dismantled and excess formulation at the surface was removed gently with absorbent tissue. The treated nail was then immediately examined by LSCM. Skin permeation experiments were performed in the same way without using the nail adaptor. The tissue was sandwiched directly between the donor and the receptor chamber (2 cm² diffusion area) and the application time was 16 hours.

![Figure 3.3: Vertical Franz diffusion cell as used in the nail permeation experiments.](image)

3.2.7 **LSCM imaging**

An LSCM 510Meta inverted laser scanning microscope (Carl Zeiss, Jena, Germany) was used. An argon laser (488 nm) and a HeNe laser (543 nm) were used to excite fluorescein and NR, respectively. The corresponding emission signals were collected using a BP 505-530 (green) and LP 560 (red) filters. Dorsal and transversal confocal images were captured to assess the localization of the fluorophores on or within the nail.
Dorsal images were acquired using an EC Plan-Neo 40x/1.3 M27 oil objective. Optical-section x-y planar images were obtained every 2 µm in z-direction. To obtain a direct transversal view of the nail, a Plan-APOCHROMAT 10x/0.45 M27 air objective was employed. The treated nail was sectioned into thin slices, which were then glued to a microscope slide with the cross-section orientated towards the objective. All transverse images were taken at a few microns below the cut surface to avoid any artefact caused by sectioning. For the skin samples, a Plan-APOCHROMAT 63x/1.4 oil DIC M27 oil objective was used for imaging and optical sectioning was performed every 1 µm into the sample. To rule out the possible interference from the nail/skin auto-fluorescence, the laser power and detector settings were minimized so that no signal was detectable from the untreated control. Reflectance and/or optical images were captured simultaneously to image the nail. All images were processed using ImageJ software (US National Institutes and Health, USA).

3.2.8 In vitro release of NR from NP suspension
The release of NR from the NPs into an oil phase, IPM, was quantified in a membrane-less system. 0.5 mL of a NP aqueous suspension was placed in an Eppendorf tube which was filled with 4.5 mL of IPM. The system was constantly stirred and the oil phase was sampled at fixed time intervals and assayed for NR content.

3.2.9 High performance liquid chromatography (HPLC)
NR was quantified using HPLC-fluorescence detector (Dionex, Sunnyvale, CA, USA). A HiQ sil C18W 250x4.6 mm column (KYA Tech, Tokyo, Japan) was employed and the mobile phase was methanol-water (95:5) at a flow rate of 1 mL/min. The excitation and emission wavelengths were set at 559 nm and 630 nm, respectively. The injection volume was 20 µL and the system was maintained at 25°C. The NR retention time was 6.5 minutes. The limits of detection and quantification were 12.7 ng/mL and 38.6 ng/mL, respectively.

3.3 Results
3.3.1 Particles characterization
The particle size and the polydispersity indices of the prepared PMMA and P(MMA-co-BMA) NPs are in Table 3.1. The size distribution profiles are illustrated in Figure 3.4.
TEM images (Figure 3.5) showed monomodal size distributions and that individual NPs were spherical and of a size that correlated well with the DLS measurements.

Table 3.1: Properties of NPs.

<table>
<thead>
<tr>
<th>NP formulation</th>
<th>Dyes</th>
<th>Mean size (nm)</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA</td>
<td>FMA and NR</td>
<td>20 ± 0.18</td>
<td>0.158 ± 0.014</td>
</tr>
<tr>
<td>P(MMA-co-BMA)</td>
<td>FMA and NR</td>
<td>22 ± 0.28</td>
<td>0.131 ± 0.008</td>
</tr>
</tbody>
</table>

Figure 3.4: Size (diameter) distribution profiles of the PMMA and P(MMA-co-BMA) NPs (n=3).

Figure 3.5: TEM images of (a) PMMA and (b) P(MMA-co-BMA) NPs. Scale bars = 50 nm.
3.3.2 LSCM imaging

FMA is a fluorescently labelled methacrylate monomer. During NP preparation, FMA reacts with MMA and/or BMA, forming homo- or co-polymers that fluoresce green when excited at 488 nm laser. NR is a lipophilic, model ‘active’ which, when excited at 543 nm, shows an intense red fluorescence. Using the multi-tracking-mode, the localization of both fluorophores was imaged simultaneously. The nail or skin surface was morphologically identified in the reflectance images.

Figure 3.6 shows the x-y planar images from nails with the dual fluorophore-labelled NPs and the NR-PG solution. Co-localization of green and red fluorescence is observed for both NP formulations with the signals decaying rapidly with increasing depth into the nail. On the other hand, NR signal is captured across the entire imaging depth in the NR-PG treated nail sample. Because of the natural curvature of the nail, the apparent penetration depths in the x-y images may not be accurate reflections of reality. For this reason, the planar images were first reconstructed into an x-z cross-sectional view. The result (Figure 3.7) indicates that all fluorescence signals from the NPs are constrained to the nail surface with no separation of NR from the NPs. In contrast, from the NR-PG solution, the fluorophore penetrated to a distance of ~100 μm into the nail. Essentially identical results were obtained from nails which were physically sectioned and examined transversally beneath the cut surface (Figure 3.8).
Figure 3.6: Planar images as a function of depth into the nail after treatment with (a) PMMA NPs, (b) P(MMA-co-BMA) NPs and (c) NR-PG solution for 7 days. Panel a1, b1 show red fluorescence from NR. Panel a2, b2 show green fluorescence from NPs. Panel a3, b3 are overlays of red and green signals. Scale bar = 50 µm.

Figure 3.7: Reconstructed cross-sectional images of nails 7 days post-treatment. Panel (a) presents the reflectance signal from the nail and the glass coverslip (above). Panel (b) illustrates NR fluorescence. Panel (c) shows the green signal of NPs. Scale bars = 50 µm.
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Figure 3.8: Images of transversally sectioned nails showing the localization of fluorophores after the 7-day permeation study. Panel (a) presents the optical micrographs of the nail. Panel (b) shows the red fluorescence from NR. Panel (c) indicates green fluorescence from NPs. Scale bars = 200 µm.

The disposition of NPs and the associated dye when applied to the skin were studied for comparative purposes. Localization of NPs and NR in skin furrows is directly observed for both PMMA and P(MMA-co-BMA) (Figure 3.9). Fluorescence from within the skin was insignificant. In contrast, the penetration of NR from a simple solution in PG into the lipid-rich intercellular domains of the stratum corneum (SC), outlining the polygonal shapes of the corneocytes, was clearly visible. Figure 3.10 illustrates the reconstructed cross-sectional views, which confirm what is seen in the x-y serial images: the NPs and their associated NR remain exclusively at the skin surface, while NR partitions into, and diffuses through, the SC when applied as a solution in PG. The minimal dissociation of the dye from the PMMA and P(MMA-co-BMA) NPs is consistent with previous findings (17).
Figure 3.9: x-y planar images obtained following 16 hours treatment of skin with NPs containing NR and with a solution of NR in PG. Panel (a) presents the reflectance signal from the skin. Panel (b) shows red fluorescence from NR. Panel (c) indicates the green signal from the NPs. Scale bars = 50 µm.

Figure 3.10: Reconstructed cross-sectional images obtained following 16 hours treatment of skin with NPs containing NR and with a solution of NR in PG. Panel (a) presents the reflectance signal from the skin and the glass coverslip (above). Panel (b) shows red fluorescence from NR. Panel (c) indicates the green signal of the NPs. Scale bars = 50 µm.
3.3.3 NR release from NPs

The release of NR from NPs into an oil phase (IPM) was studied. However, even after 7 days, the amount of NR found in the receptor medium was below the limit of detection (Figure 3.11). Despite good solubility of NR in IPM, it is clear that the dye preferred to remain with the NPs.

![Figure 3.11: Illustration of NR (non)-release from PMMA (left) and P(MMA-co-BMA) (right) NP suspensions into IPM. The photo was taken at the end of the 7-day experiment.](image)

3.4 Discussion

The results confirm that the NPs studied, despite their rather small diameters of only ~20 nm, are unable to penetrate into the nail or the skin. There is simply insufficient free volume, either trans- or inter-cellularly, to allow translocation to occur (18-21). Such polymeric NPs cannot, therefore, act as drug ‘carriers’ across either the skin or nail.

The potential exists, however, for the methacrylate-based NPs to act as drug reservoirs for topical delivery to the nail. This was explored here using LSCM to track the disposition of a model, lipophilic ‘active’ (NR) after topical application of two NP formulations. Unfortunately, NR separation from the NPs did not occur (as was also the case in simple NR release experiments) suggesting a very high affinity of the dye for the complex interplay of various parameters, including the molecule’s physicochemical properties, its interaction with the polymer, how it distributes within the system (adhesion to the surface, entrapment in the polymer matrix or combination of both), properties of the NPs themselves (hydrophobicity, hydrophilicity, rigidity and biodegradability) and the solubility of the stabilizing surfactant. The negligible amount
of NR release obtained here may be a consequence of several factors. For example, the highly hydrophobic nature of the polymers may serve as a good solvent for NR (22), resulting in a very low leaving tendency. It is also possible that the relatively high glass transition temperatures ($T_g$) of PMMA and P(MMA-co-BMA 50/50) (103 °C and 50°C, respectively (15)) renders their solid cores too rigid for ‘leakage’ of the entrapped NR to occur. Furthermore, the presence of the anionic surfactant, SDS, may create an electrical double layer (23) around the NPs conferring high stability to the suspension and inhibiting the partitioning out of NR into the surrounding environments.

### 3.5 Conclusions

Small (~20 nm) PMMA and P(MMA-co-BMA) NPs when applied to the nail for a period of 7 days remained on the surface. The same behaviour was seen on skin, albeit for a shorter exposure time (16 hours). The potential value of the NPs as reservoirs from which to sustain drug delivery over a prolonged period could not be determined, however, because of the high affinity of the lipophilic ‘active’ for the particles used in this work. It follows that further work is required using less hydrophobic actives and modifying the NP polymer properties to lower the glass transition temperature closer to, or even below, the physiological value.
3.6 References


Chapter 4: Delivery of an active from nanoparticles into microneedle-treated nail

Overview

Purpose: To demonstrate the sustained release of an active from poly(ε-caprolactone) nanoparticles (NPs) into microneedle-treated human nail.

Methods: NPs were prepared and loaded with a model active, the lipophilic fluorophore, Nile red (NR). NP size and morphology were determined by dynamic light scattering and transmission electron microscopy, and the in vitro release of NR from a NP suspension was characterized. The disposition of NR following topical application to intact nail and to nail porated with microneedles was imaged using laser scanning confocal microscopy (LSCM) as a function of time over 7 days.

Results: The polymeric NPs were spherical with diameters of ~150 nm. Post-application to the nail, sustained release of NR from the NPs were observed. Confocal imaging clearly showed NR localization between the nail plate cells. Microneedle-created pores shortened the penetration pathway of the active across the nail, and permitted lateral diffusion of NR to occur more rapidly into the deeper regions of the nail.

Conclusions: Sustained NR delivery into the nail from polymeric NPs was achieved. The model active penetrated the nail via an intercellular penetration route. When the nail was pre-treated with microneedles, NR delivery was clearly enhanced. The NPs appeared to accumulate both on the nail surface and within the microneedle-generated pores, from which the loaded active was released and diffused laterally into the nail.
4.1 Introduction

Biocompatible and biodegradable polymeric nanoparticles (NPs) have been proposed as constituents of topical formulations to act as drug reservoirs from which sustained delivery into the skin and increased local bioavailability can be achieved (1-3). The objective of the research described in this paper is to examine whether this strategy might be applied to improve the treatment of nail diseases. On the skin, the highly convoluted surface, and the entrances to appendageal structures, such as follicles, offer sites at which NPs can accumulate with prolonged residence times during which release of their drug payload is possible. In contrast, the nail surface has a less dramatic topography and is self-evidently not perforated by hairs or sweat glands. To provide sites in the nail into which drug-loaded NPs can be sequestered, pre-treatment with a microneedle array is considered here. This ‘poration’ approach has, of course, been the subject of intensive study with respect to drug delivery into and through the skin, where piercing the stratum corneum creates new, low-resistance pathways through which the active may gain facile and rapid access to the underlying viable epidermis (and, ultimately, the systemic circulation) (4). While it is unlikely that a microneedle array can completely penetrate through the nail, the opportunity exists to create fissures into which NPs may be deposited, and from which a prolonged and slow release of an active can then occur.

In this study described below, NPs were prepared from poly(ε-caprolactone) (PCL), a hydrophobic, biocompatible and biodegradable semi-crystalline polymer. Drug release from these NPs is generally efficient due to the polymer’s low glass transition temperature ($T_g = -60^\circ C$). For this reason, PCL has been frequently used in the formation of colloidal vectors (5), and NPs prepared from this polymer are stable (6), have successfully encapsulated various drugs, and have sustained and enhanced drug delivery into the skin (7-9). Here, NPs were synthesized from PCL using a solvent displacement (or nanoprecipitation) method (10). This is a simple, reproducible method, which has been widely adopted for the production of nanospheres or nanocapsules. Nile red (NR), acting as a lipophilic model drug, was incorporated into the NPs, and laser scanning confocal microscopy (LSCM) was employed to visualize the distribution of NR following the topical application of NPs to untreated and microneedle-porated nails.
4.2 Materials and methods

4.2.1 Chemicals
Preformed polymer PCL (MW: 10,000), polysorbate 85 (Tween 85), NR (analytical grade), propylene glycol (PG) and isopropyl myristate (IPM) were obtained from Sigma-Aldrich Co. (Gillingham, UK).

4.2.2 Nail sample preparation
Human fingernail clippings (at least 8 mm in length) were obtained from healthy volunteers who gave informed consent. Ethical approval was granted by the Research Ethics Approval Committee for Health (REACH; EP 11/12 115) of the University of Bath (Forms in Appendix 1). Nails were maintained frozen (-20°C) until use. Prior to the experiments, the nail was soaked in deionized water for 30 minutes to restore some flexibility. Microporation was performed using a commercially available dermaroller (Infinitive beauty®, Birmingham, UK), comprising 250 µm long titanium needles, which was applied to the dorsal side of the hydrated nail by rolling it back and forth a total of 5 times.

4.2.3 Nanoparticle preparation
NR-loaded PCL NPs were prepared by solvent displacement method (7). An acetone solution (10 mL) containing 5 mg of NR and 125 mg of PCL was injected drop-wise into 50 mL of an aqueous solution containing 3% (w/v) of Tween 85. NPs were formed rapidly, creating an opalescence suspension. The acetone was subsequently removed under reduced pressure and the NP suspension was further concentrated to a final volume of 20-30 mL, which was then filtered (0.45 µm nylon filters, Whatman, Maidstone, UK) to remove any un-encapsulated precipitated aggregates.

4.2.4 Nanoparticle characterization
The nanoparticle size and polydispersity index were measured (n=3) using dynamic light scattering (ZetaSizer Nano S, Malvern, UK), following a 100-fold dilution of the suspension. NP morphology was imaged using a JEOL JEM-2000 transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 120 kV. Each specimen was prepared by drop-casting the NP suspension onto a carbon coated copper grid, which was then cryodessiccated before imaging.
The amount of NR entrapped in the NPs was calculated from the difference between the quantity found after complete dissolution of the particles in acetonitrile ($Q_{\text{tot}}$), and that in the aqueous supernatant ($Q_{\text{sup}}$) after ultrafiltration-centrifugation of the suspension at 6000 rpm for 30 minutes (MWCO 3000, Millipore Corp., Watford, UK). All experiments were performed in triplicate. The entrapment efficiency (%) was given by: 
\[
100 \times \left\{ \frac{(Q_{\text{tot}} - Q_{\text{sup}})}{Q_{\text{input}}} \right\},
\]
where $Q_{\text{input}}$ represents the amount of NR used in the preparation process.

### 4.2.5 NR release

NR release from the NPs into an oil phase was quantified in a membrane-less system. 0.5 mL of an aqueous NP suspension was placed in an Eppendorf tube and 4.5 mL of isopropyl myristate (IPM) was added. The system was constantly stirred and the oil phase was sampled at fixed time intervals. Subsequently, the samples were assayed for NR content. The release of NR from a solution in PG (at the equivalent NR concentration as in the NP suspension) was performed as a positive control. To obtain the solubility of NR in IPM, a saturated solution was prepared by stirring excess solute into the solvent over a period of 48 hours. The final suspension was then filtered through a nylon membrane (pore size = 0.45 µm) and the concentration of NR in the filtrate was quantified (n=3).

### 4.2.6 High performance liquid chromatography (HPLC)

NR was quantified using HPLC-fluorescence detector (Dionex, Sunnyvale, CA, USA). A HiQ sil C18W 250 x 4.6 mm column (KYA Tech, Tokyo, Japan) was employed and the mobile phase was methanol-water (95:5) at a flow rate of 1 mL/min. The excitation and emission wavelengths were set at 559 nm and 630 nm, respectively. The injection volume was 20 µL and the system was maintained at 25°C. The NR retention time was 6.5 minutes. The limits of detection and quantification were 12.7 and 38.6 ng/mL, respectively.

### 4.2.7 In vitro nail permeation

NR uptake into non-porated nail was first evaluated in vertical Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA, diffusion area = 0.2 cm²). The nail was mounted with the dorsal surface facing the donor chamber; whereas the ventral side was fully hydrated by contact with phosphate buffer saline (pH 7.4) in the receptor chamber.
300 µL of the NP suspension was then introduced into the donor compartment, which was covered with Parafilm® to avoid evaporation. The entire diffusion cell was then placed in an incubator at 32 °C. Nails treated with deionized water served as negative controls. After 7 days, the diffusion cell was dismantled and excess formulation at the surface was removed gently with absorbent tissue. The nail was then immediately examined by LSCM.

NR uptake as a function of time into microneedle-porated nail was then accessed after application of 10 µL of the NP suspension onto the dorsal surface of the samples, which were then sandwiched between two glass coverslips and sealed securely with double-sided tapes and Parafilm® to avoid evaporation. Confocal images of selected regions of interest were then recorded as a function of time. Identical control experiments were performed using untreated nails.

4.2.8 LSCM imaging

An inverted laser scanning microscope (510Meta, Carl Zeiss, Jena, Germany) was used. A HeNe laser (543 nm) was employed to excite NR and the emission signals were collected using a LP 560 filter. Planar (x-y) and cross-sectional confocal images were captured to assess the localization of the fluorophore on or within the nail. Dorsal images were acquired using an oil objective (EC Plan-Neo 40x/1.3 M27). To obtain a direct transverse view of the nail, an air objective (Plan-Apochromat 10x/0.45 M27) was employed. In this case, the treated nail was thinly sectioned and the slices were then glued to a microscope slide with the cross-section orientated towards the objective. All transverse images were recorded from a few microns below the cut surface to avoid any artefact caused by the sectioning. To rule out interference from the nail auto-fluorescence, the laser power and detector settings were minimized so that no signal was detectable from the untreated control. Reflectance and/or optical images were captured simultaneously to image the nail.

4.2.9 Data analysis

All confocal images were processed using ImageJ software (US National Institutes and Health, USA). Fluorescence signal quantification at various nail depths was performed using the ‘specify’ and then ‘plot profile’ plugins in ImageJ.
4.3 Results and discussion

4.3.1 Nanoparticle characterization

The average NP diameter was 152 ± 3 nm with a relatively small polydispersity index of 0.149 ± 0.0076 (Figure 4.1); the individual particles were spherical (Figure 4.2). The encapsulation efficiency of NR in the polymeric NPs was 89 ± 0.76%, a result consistent with the other studies (13, 14), which have reported the high loading efficiency of lipophilic compounds. No free NR was detected in the suspension presumably due to the compound’s very low water solubility. The final NR concentration in the nano-suspension was 206 ± 2 µg/mL.

Figure 4.1: Size (diameter) distribution profiles of the NR-loaded PCL NPs (n=3).

Figure 4.2: TEM images of NPs of PCL, loaded with NR. Scale bars = 100 nm.

4.3.2 In vitro release studies

The solubility of NR in IPM was found to be 379 ± 7.7 µg/mL and the concentrations measured in the release study were always less than one-tenth of this value confirming that sink conditions were maintained. The cumulative (%) release of NR from a solution in PG, and from a polymeric NP suspension, as a function of time is shown in Figure
4.3. From the former, 80% is released within 3 hours; for the NPs, this level of release was achieved only after 12 hours. The release of NR from the NPs appears to be biphasic, with a faster component during the first 3 hours (attributable to the relatively rapid dissociation of surface-bound NR), followed by a slower phase controlled presumably by diffusion of the entrapped marker from within the polymer matrix. Figure 4.4 provides a clear visual representation of NR release from the NPs at early and late times during the experiment. These results may be contrasted with the previously reported effective non-release of NR from NPs based on poly-methacrylate, a polymer with much higher glass transition temperature ($T_g$) than PCL. In addition, the difference may also be influenced by the size and NR-loading differences between the two types of NPs.

![Graph showing cumulative NR released vs time](image)

**Figure 4.3:** *In vitro* release of NR from a solution in PG, and from a polymeric (PCL) NP suspension, as a function of time (mean ± SD, n=3).

![Images showing NR release](image)

**Figure 4.4:** Visualization of NR release from a PCL NP suspension into IPM after 0.5 hours (left) and 12 hours (right).
4.3.3 LSCM imaging

**In vitro uptake of NR into non-porated nails**

The distribution of NR on or within the non-porated nail after post-application of the NP suspension for 7 days was examined using LSCM. Images from 3 representative nail samples of 3 different volunteers are shown in Figure 4.5 and Figure 4.6. The former shows cross-sectional (x-z) reconstructions of the sequentially acquired planar (x-y) images; both reflectance and fluorescence signals are presented. An intense, bright red band of fluorescence from NR is observed on and within the superficial part of the nail, with a measurable signal perceptible down to depths of approximately 70-90 µm. These observations are completely consistent with the direct, transverse visualization of mechanically sectioned nail slices depicted in Figure 4.6. It follows that, over 7 days, NR was released from the NPs and successfully permeated into the nail plate to a depth corresponding to about 30% of its total thickness. Furthermore, the results are completely consistent with the ~150 nm diameter NPs being unable themselves to transport across the densely interdigitated onychocytes.

![Figure 4.5: Reconstructed cross-sectional images obtained following 7 days of permeation. Panel (a): reflectance signal from the nail plate. Panel (b): NR fluorescence. Scale bar = 50 µm.](image)

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Figure 4.6: Illustrative images of 3 transversally sectioned nails: (a) optical micrographs, (b) NR fluorescence, and (c) the merged images. Scale bar = 200 μm.

The transport pathway of NR across the nail may also be identified. Figure 4.7 (and as videos in Supplementary data 4.1a,b) provides illustrative x-y planar images of an example, showing the reflectance signal and NR fluorescence at approximately 10 μm, 20 μm and 30 μm depths into the nail. The reflectance images reveal the fairly regular polyhedral, squamoid shape of the onychocytes that measure about 30 μm across (11, 12). Localization of NR at the intercellular regions was clearly visible, a point reinforced by the simultaneously acquired fluorescence images. As expected, the hydrophobic NR has high affinity for these more lipid-rich domains, even though the total lipid content of the nail plate is low (0.1-1%) (13). Indeed, the permeation of lipophilic compounds has been considered unfavourable, despite suggestions that transport, in fact, has little dependence on a drug’s lipophilicity (14, 15), and the identification of transport pathways remains an open question. Nonetheless, nail plate is known to contain phospholipid particularly in the dorsal and ventral layers (16), and the intercellular spaces (25-35 nm) are reported to be filled with a ‘mortar’ that contains membrane-coating granules comprised primarily of phospholipids (17). These domains
may therefore provide a sympathetic environment for small, hydrophobic molecules and constitute a potential lipophilic pathway for nail permeation. Bright (i.e., NR-stained) punctate features were also observed, increasing in number with depth into the nail and may correspond to nuclear remnants (retained shrunken or fragmented nuclei), also known as ‘pertinax bodies’ (18).

Figure 4.7: Planar images (x-y) of NR disposition in a nail at depths 10, 20 and 30 μm: (A) reflectance signal; (B) NR fluorescence. Scale bars = 50 μm.

**In vitro uptake of NR into non-porated and porated nails as a function of time**

Subsequently, the disposition of NR following application of a PCL NP suspension to both non-porated and microneedle-porated nails was assessed as a function of time; the results are visualized in Figure 4.8. In intact nails, there remains the most intense signal at the surface where the NP formulation is localized; however, as time progresses, release and penetration of NR produces a slowly increasing level of fluorescence in deeper regions of the nail.

The impact of microneedle poration is self-evident from the distinctly different images seen in Figure 4.8b. First of all, when the microneedle device is rolled back and forth over the nail surface, not only are rectangular-shaped pores created, but unavoidable scratch marks are also made (as can be seen in the images). While there is still intense NR fluorescence at the nail surface, there is also a large signal from within the pores, at short times post-application, demonstrating that the NPs themselves are localized here. Images acquired at later time points reveal both the release and penetration of NR from
the NP ‘reservoirs’ on the surface and within the pores, but also the enhanced lateral diffusion of the fluorophore from the sides of the pores. As observed in the 7-day experiment on non-porated nails, intercellular NR penetration is apparent in nails that have been microneedle-porated. As hypothesized, therefore, the microneedle-created pores in the nail fulfil a similar role to that described for skin furrows and hair follicles when NP formulations are applied topically (19), in addition to shortening the diffusion path-length of the penetrant once released from the carrier.

The reconstructed x-z orthogonal image of a non-porated nail (Figure 4.9) recorded 48 hours after the start of the experiment reveals an intense signal from the NP formulation at the surface and that NR, which has been released, has penetrated ~25 µm into the nail plate. In contrast, the corresponding images from a microneedle-porated nail (Figure 4.9) shows not only the concentration of NPs at the surface but that they have also filled the pore and gained access to the nail down to a depth of ~70 µm. Moreover, a red ‘haze’ in the surrounding nail tissues is clearly observed indicating that enhanced, lateral NR release has taken place.

Figure 4.10 provides semi-quantitative support for this conclusion and presents the average fluorescence signal across the orthogonal images as a function of depth into the intact and porated nails. While the signal at 0-10 µm originates primarily from the superficial formulation layer in both samples, the fluorescence rapidly drops to baseline with increasing depth into the intact nail. The signal profiles from the porated nail manifest the strong fluorescence peaks within the pore. While the regions distant from the pore show rapid signal decay with depth, those close to the pore reveal the presence of a more substantial fluorescence, confirming the evident lateral diffusion of NR.
Figure 4.8: LSCM visualization of NR disposition as functions of time and depth into (a) the intact and (b) the microneedle-porated nail (shown in the reflectance images). Scale bars = 50 µm.
Figure 4.9: Reconstructed cross-sectional images recorded 48 hours after application of NR-loaded PCL NPs to intact and microneedle-porated nails as a function of depth. Panel (a): reflectance signal from the nail plate. Panel (b): NR fluorescence. Scale bars = 50 μm.

Figure 4.10: NR fluorescence signal intensity profiles recorded 48 hours after the start of the experiment across the orthogonal images of intact and microneedle-porated nails as a function of depth. Grey highlighted areas indicate regions immediately surrounding the pores.

Overall, the results of this study lead to the model, by which NPs deliver their payload into microneedle-porated nail, shown in Figure 4.11. The long residence time of NR fluorescence on the nail surface and within the pore attests to the non-penetrability of the NPs and themselves across the barrier, as has been inferred in a number of publications reporting their ‘fate’ on the skin (19-22). The diffusion of fluorescence, increasing with time and distance from the surface/pore supports the release of NR from the NPs and its subsequent penetration into the nail. Absolute confirmation, nonetheless, requires both particle and penetrant to be simultaneously and unambiguously tracked, a challenge that will be addressed in the next chapter of this thesis.
4.4 Conclusions

A lipophilic fluorophore, Nile red (NR), has been encapsulated into polymeric nanoparticles (NPs). Subsequently, the disposition of NR following topical application to intact nail and to nail porated with microneedles was imaged using laser scanning confocal microscopy for 7 days. Post-application to the nail, sustained release of NR from the NPs was observed. Confocal imaging clearly showed NR localization between the nail plate cells. Microneedle-created pores (a) created sequestration sites for the NPs from which NR release continued throughout the experiment, and (b) permitted lateral diffusion of NR to occur more rapidly into the deeper regions of the nail. To substantiate these conclusions, further experiments are required in which both particle and penetrant can be simultaneously and unambiguously tracked.
4.5 References

Chapter 5: Disposition of nanoparticles and an associated lipophilic active into micro-porated nail: imaging using stimulated Raman scattering and two-photon fluorescence microscopy

Overview

Purpose: To characterize the release of a lipophilic active from poly(ε-caprolactone) nanoparticles (NPs) into microneedle-porated human nail using stimulated Raman scattering and two-photon fluorescence microscopy.

Methods: Poly(ε-caprolactone) was synthesized from ε-caprolactone and covalently labelled with a fluorescent tag. NPs were prepared from this polymer and loaded with a model lipophilic ‘active’, octyl methoxycinnamate (OMC). The NPs were applied to human nails that had been microneedle-porated and two-photon fluorescence (TPF) and simulated Raman scattering (SRS) were used to visualize the distribution of the particles and OMC, respectively, after 3 hours and 7 days of application.

Results: The stability of the covalent linkage between the polymer and the fluorescent tag was confirmed. The NPs were spherical with an average diameter of 135 nm. A high entrapment efficiency of OMC (96 ± 1.95%) into the NPs was achieved. Co-localization of NPs and OMC on the nail surface and within the ‘trenches’ created by the microneedles was observed 3 hours post-application. After 7 days, however, OMC had been progressively released from the NPs and had penetrated deeper and laterally into the nail. The location of the NPs, in contrast, remained unchanged.

Conclusions: The independent visualization of the NPs using TPF, and of the model active by SRS, provides unambiguous evidence that the polymeric particles act as drug reservoirs, confined to the nail surface and to the microneedle-created pores, from which sustained release of the associated active can be achieved over a period of several days.
5.1 Introduction

The nail plate is an effective barrier that significantly restricts the permeation of drugs and results in the fact that current topical therapy to treat nail disease is often unsuccessful. To address this unmet clinical need, a number of strategies are under investigation to improve drug delivery into and through the nail, including chemical penetration enhancers (1, 2) and iontophoresis (3, 4). A novel colloidal formulation approach using polymeric nanoparticles (NPs) has been assessed in this work. A key component of the research is visualization of the disposition of the NPs and their associated ‘active’ payload following topical application to the nail. The resulting information is anticipated to provide useful support for the further development and optimization of topical nail formulations.

It has been previously demonstrated that laser scanning confocal microscopy is a valuable imaging technique to track the distribution of fluorescent compounds in the nail. However, to follow unambiguously the disposition of both the active and the NP carriers requires two fluorophores with distinct excitation and emission spectra. Furthermore, the range of available fluorophores is far from representative of typical drugs (for example, in terms of their structural and physicochemical properties) and extrapolation of their behaviour to the therapeutic situation must be undertaken with caution.

Stimulated Raman scattering (SRS) microscopy is an imaging tool with which to tackle this problem. SRS is a label-free, non-destructive imaging technique based on molecular vibrational spectroscopy, and its high-resolution 3D imaging capability has led to application in a wide range of research areas (5). The use of SRS to assess solvent diffusion into the nail plate has been described in Chapter 2. The technique has also been employed, together with fluorescence microscopy, to visualize skin structure (6), and the uptake of drug into fluorescently-tagged lysosomes in living cells (7). Here, SRS and two-photon fluorescence (TPF) imaging are used in combination to probe the fates of poly(ε-caprolactone) (PCL) NPs and an associated lipophilic ‘active’ in microneedle-porated human nails.

5.2 Materials and methods

5.2.1 Chemicals

ε-caprolactone, polysorbate 85 (Tween 85), octyl methoxycinnamate (OMC) and tin(II) 2-ethylhexanoate (stannous octoate) were obtained from Sigma-Aldrich Co.
5.1 (Gillingham, UK). 5,10,15,20-tetrakis-(4-aminophenyl)porphyrin (TAPP) was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan).

5.2.2 Nail sample preparation

Human fingernail clippings (at least 8 mm in length) were obtained from healthy volunteers who gave informed consent. Ethical approval was granted by the Research Ethics Approval Committee for Health (REACH; EP 11/12 115) of the University of Bath (Forms in Appendix 1). Nails were maintained frozen (-20°C) until use. Prior to the experiments, the nail was soaked in deionized water for 30 minutes to restore some flexibility. Microporation was performed using a commercially available dermaroller (Infinitive beauty®, Birmingham, UK), comprising 250 µm long titanium needles, which was applied to the dorsal side of the hydrated nail by rolling it back and forth a total of 5 times.

5.2.3 Synthesis of TAPP-labelled PCL

Star-shaped PCL with a TAPP core was synthesized (Figure 5.1) (8). TAPP (0.025 mmol), stannous octoate (0.0025 mmol) and ε-caprolactone (0.7 g) were introduced into a 10 mL round-bottom flask under argon and the polymerization process was carried out with stirring at constant temperature (110°C) for 72 hours. When the reaction was completed, the cooled product was dissolved in dichloromethane, and subsequently precipitated by drop-wise addition to cold stirring methanol. The precipitate (TAPP-PCL) was filtered and dried at 40°C. 1H NMR spectra of the synthesized TAPP-PCL in deuterated chloroform (CDCl₃) was recorded on a Bruker Avance™ III spectrometer (Billerica, MA, USA) operating at 400 MHz.
5.2.4 Nanoparticle preparation

Fluorescently-labelled NPs loaded with OMC were prepared using the synthesized TAPP-PCL. An organic phase comprising OMC (5.1% w/v) and TAPP-PCL (125 mg) in 10 mL of acetone was injected slowly into 50 mL of an aqueous solution of Tween 85 (3% w/v), forming a turbid suspension. The acetone was subsequently removed under reduced pressure and the NP suspension was further concentrated to a final volume of ~20-30 mL, which was then filtered (0.45 µm nylon filters, Whatman, Maidstone, UK) to remove any precipitates.

5.2.5 Nanoparticle characterization

The nanoparticle size and polydispersity index were measured (n=3) using dynamic light scattering (ZetaSizer Nano S, Malvern, UK), following a 100-fold dilution of the suspension.

NP morphology was imaged using a JEOL JEM-2000 transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 120 kV. Each specimen was prepared by drop-casting the NP suspension onto a carbon coated copper grid, which was then cryo-desiccated before imaging.

The amount of OMC entrapped in the NPs was calculated from the difference between the quantity found after complete dissolution of the particles in acetonitrile ($Q_{\text{tot}}$), and that in the aqueous supernatant ($Q_{\text{sup}}$) after ultrafiltration-centrifugation of the suspension at 6000 rpm for 30 minutes (MWCO 3000, Millipore Corp., Watford, UK).

All experiments were performed in triplicate. The entrapment efficiency (%) was given by: \( \{100 \times [(Q_{\text{tot}} - Q_{\text{sup}}) / Q_{\text{input}}]\} \), where $Q_{\text{input}}$ represents the input amount of OMC.
OMC concentration was quantified using high performance liquid chromatography (HPLC) (Jasco, Great Dunmow, UK) with UV detection at 310 nm. A mobile phase consisting of acetonitrile-water (85:15) at pH 2.5 (adjusted using orthophosphoric acid) was pumped at 1 mL/min through a Acclaim® C18 (5 µm) 150x4.6 mm column. Each injection was 10 µL and the retention time was 9.5 minutes. The limits of detection and quantification were calculated as 2.6 µg/mL and 7.9 µg/mL, respectively.

5.2.6 Raman spectroscopy
The Raman spectra of OMC and of a human nail sample were recorded (Renishaw RM1000 Raman microscope and v1.2 WIRE software, Renishaw plc, Wotton-Under-Edge, UK). A 1200-line/mm grating providing spectral resolution of 1 cm\(^{-1}\) was used with a diode laser exciting at 785 nm with up to 300 mW power. The Raman band (520 cm\(^{-1}\)) of a silicon wafer was used for calibration. The Vancouver Raman Algorithm (BC Cancer Agency & University of British Columbia, Canada) permitted automated removal of fluorescence background from the Raman spectra.

5.2.7 SRS and TPF imaging
The distribution of OMC and TAPP-PCL NPs on and within the microneedle-porated nails after either a 3-hour or 7-day application was determined. The nails were mounted in vertical Franz diffusion cells (see Chapter 4) and 10 µL of an aqueous NP suspension was applied to an area of 0.2 cm\(^2\). Before imaging, the samples were sandwiched between two glass coverslips and sealed securely with double-sided tapes and Parafilm® to avoid evaporation. The central areas of the nails were subsequently imaged. SRS and TPF imaging were carried out sequentially using a custom-built, multi-modal, inverted microscope with a confocal laser scanner (FV300/IX171, Olympus UK Ltd, UK).

Synchronized, dual-wavelength picosecond excitation was provided by an optical parametric oscillator (OPO) (Levante Emerald, APE, Berlin) which was synchronously pumped at 532 nm by a frequency-doubled Nd:Vanadium laser (picoTRAIN, High-Q GmbB), delivering a 7 ps pulse train at a 76 mHz repetition rate. The OPO consisted of a temperature-tuned, non-critically phase matched Lithium Triborate (LBO) crystal, which allows the OPO signal (employed as the pump beam) to be continuously tuned from 690 to 980 nm by adjusting the LBO temperature and an inter-cavity Lyot filter. A Si PIN photodiode was used to record the intensity variations of the OPO signal. The
pump-laser fundamental (1064 nm) was also available as a separate output and was used as the Stokes beam, which was amplitude modulated at 1.7 MHz with an acousto-optic modulator (3080-197 Crystal Technologies, West Chester, PA, USA). The pump beam and the modulated Stokes beam were spatially overlapped using a dichroic mirror (1064 DCRB, Chroma Technology Corp, Bellows Falls, USA) and temporally overlapped using a delay stage. The collinear beams were directed into the microscope and focussed onto the sample using a 60x 1.2 NA water immersion objective (UPlanSApo, Olympus) and scanned in two dimensions using a pair of galvanometer mirrors. The resulting SRL in the pump beam was collected in the forward direction via a 1.0 NA condenser lens (LUMFI, Olympus) and detected by a large area photodiode (FDS1010, Thorlabs, New Jersey, USA). A band-pass filter (850/90 nm, Chroma) was mounted in front of the detector to block the modulated 1064 nm beam. Finally, a lock-in amplifier (SR844, Stanford Research Systems, Sunnyvale, CA, USA) was used to detect the SRL signal with a time constant of 30-100 μs.

TPF was excited using the OPO signal output at 816 nm with the 1064 nm beam shuttered. The signal was collected in the epi-direction and detected by a photomultiplier tube (R3896, Hamamatsu, Hertfordshire, UK). The absence of nail autofluorescence detected with the laser and detector settings used in the experiment was confirmed by imaging a control, untreated nail sample.

5.2.8 Image analysis
All images acquired were processed using ImageJ (US National Institutes and Health, USA). Each SRS data point was normalized against the OPO signal recorded on the PIN photodiode to correct for the laser intensity fluctuations. Images of different Raman shifts and/or fluorescence signal were presented using different colour schemes for ease of interpretation.

5.3 Results and discussion
5.3.1 Fluorescently-tagged nanoparticle synthesis
The reactant fluorophore, TAPP acted as an initiator during the stannous octoate-catalyzed polymerization of e-caprolactone. The $^1$H NMR spectrum of the TAPP-PCL is shown in Figure 5.2, the features of which clearly indicate that TAPP was covalently bound to PCL. This was essential for interpretation of the TPF imaging, confirming that
the detected TAPP fluorescence originated only from NPs, the disposition of which could therefore be tracked unambiguously.

Figure 5.2: $^1$H NMR spectrum of TAPP-PCL.

### 5.3.2 Particles characterization

The TAPP-PCL NPs entrapped OMC, a lipophilic UV filter commonly used in topical sunscreen products. OMC was chosen because it is known to be efficiently loaded into polymeric NPs (9-11) due, at least in part, to its high lipophilicity (average LogP = 5.46 (12)). Furthermore, OMC is a liquid in room temperature and can act itself as the oil phase in the nanoprecipitation process. The average NP diameter was $136 \pm 1$ nm with a polydispersity index of $0.26 \pm 0.01$ (Figure 5.3); individual particles were spherical (Figure 5.4). The final OMC concentration in the suspension was $17.8 \pm 0.4$ mg/mL and the encapsulation efficiency was $96 \pm 2\%$. 


5.3.3 Raman spectroscopy

The chemical structure of OMC and the Raman spectra of the chemical and of a nail sample are shown in Figure 5.5. In the OMC spectrum, the absorbance centred at 1600 cm\(^{-1}\) originates from the aromatic C-C stretching. In contrast, there is little or no signal at this frequency in the nail spectrum. Although TAPP may also be expected to contribute to a SRS signal at 1600 cm\(^{-1}\), the fluorophore can be uniquely identified and differentiated from OMC using TPF.
Figure 5.5: Chemical structure of OMC (left), and the Raman spectra of the chemical and of a nail sample (right). The arrow indicates aromatic C-C bond stretching from OMC at 1600 cm\(^{-1}\).

5.3.4 SRS and TPF imaging

The disposition of OMC and TAPP-PCL on and within the microneedle-porated nail following application of the NP suspension for 3 hours and 7 days were imaged by SRS and TPF microscopies. SRS was tuned sequentially to 2855 cm\(^{-1}\) to image (principally) the -CH\(_2\) bond stretching vibration from the nail, 1802 cm\(^{-1}\) to record any off resonance background, and 1600 cm\(^{-1}\) to image the aromatic C-C stretch from OMC. TPF microscopy was used to locate the fluorescently-tagged NPs. The x-y planar images (236 \(\mu\)m x 236 \(\mu\)m) were captured in 2 \(\mu\)m depth increments (but are shown only every 4 \(\mu\)m to economize on space). The scan time for each stack was 18.4 seconds.

Although -CH\(_2\) signal originated uniquely from the nail in the previous study examining the uptake of perdeuterated solvents (Chapter 2), this assignment is less specific here because both OMC (as shown in Figure 5.5) and the PCL polymer also include -CH\(_2\) groups in their structures and contribute therefore to this resonance.

The off-resonance signal verified that there was essentially no background when the laser was tuned away from the target vibrational resonances. Only occasional light scattering from small residual dirt particles on the nail surface and an extremely low endogenous signal from the nail were detected.

The aromatic C-C signal could have originated from both OMC and TAPP, as mentioned before, but the TPF signal allowed specific and unequivocal imaging of the NPs.

Figure 5.6 shows the x-y planar images of SRS and TPF signals obtained at different depths into the nail pre-treated with a microneedle roller. The rectangular, ‘trench-like’ structures created are clearly visible. At 3 hours post-application, the images confirm
the co-localization of the aromatic C-C signal (OMC) and the TAPP fluorescence on the nail surface and within the pores. The overlaid images reveal essentially perfect alignment of the signals at all imaging depths and that minimal separation of OMC from the NPs is apparent on this short time-scale.

After 7 days, on the other hand, while the fluorescence from the NPs remains exclusively confined to the nail surface and within the pores, the SRS signal from OMC indicates its release from the NPs and subsequent diffusion away from the surface/pore into the nail itself.

Figure 5.7 shows the three-dimensional reconstruction of a representative microneedle-created ‘trench’. The fluorescence signal from the NPs is visibly intense on the nail surface and within the internal confines of the ‘trench’, but no movement of the NPs beyond these boundaries has occurred. In contrast, the diffusion of OMC (denoted by the SRS aromatic C-C signal) into the nail is clearly visualized, supporting the separation of the active from the NPs and its permeation into and through the nail.
Figure 5.6: SRS (panel A-C) and TPF (panel D) images recorded as a function of depth after (a) 3 hours, and (b) 7 days application of OMC-loaded, fluorescently-tagged NPs to microneedle-porated nails. (A) -CH$_2$ resonance primarily from the nail. (B) Off-resonance signal. (C) Aromatic C-C vibration from OMC. (D) TPF from the NPs. (E) Overlay of panels B and C. Scale bar = 50 µm.
Figure 5.7: Three-dimensional reconstruction (prepared using ImageJ ‘volume viewer’ plugin) of a representative microneedle-created pore, 7 days post-application of a NP suspension onto the porated nail, showing the distribution of (A) -CH₂ resonance primarily from the nail. (B) TPF from the NPs. (C) Aromatic C-C signal from OMC. (D) Overlay of panels B and C. Scale bar = 50 μm.

As reported before, the microneedle-porated ‘trenches’ provide sites into which NPs can accumulate. Taken together, the SRS and TPF images clearly confirm that, 7 days post-application, OMC has been released into, and has started to diffuse within, the nail while the fluorescently-tagged NPs remain confined to the surface and within the pores. These results concur fully from the observations reported in the previous chapter where the release of a fluorescent ‘active’ from unlabelled NPs was inferred from confocal microscopy imaging. The data presented here confirm this deduction unambiguously: the NPs do indeed act as immobile reservoirs, sequestered on the nail and in the physically created pores, sustaining the prolonged release of an active payload over an extended period of time.

5.4 Conclusions
The release of octyl methoxycinnamate (OMC), a lipophilic active, from fluorescently-labelled poly(ε-caprolactone) nanoparticles (NPs) into microneedle-porated human nail

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using stimulated Raman scattering and two-photon fluorescence microscopy has been demonstrated. While co-localization of NPs and OMC on the nail surface and within the ‘trenches’ created by the microneedles was observed a few hours post-application, after one week, OMC had been released from the NPs permeating deeper and laterally into the nail. Over the same time, in contrast, no movement of the NPs was perceptible. The results provide clear evidence that the NPs function as reservoirs for lipophilic compound and sustain its release over a period of several days.
5.5 References


Chapter 6: Release and nail permeation of ciclopirox in vitro from conventional and sub-micron particle formulations

Overview

Purpose: To investigate the release and nail permeation of antifungal drug, ciclopirox (CPX), from lacquer and sub-micron particle-based formulations and to evaluate the effects of nail pre-treatment on drug uptake.

Methods: The release and nail permeation of CPX from a commercially available lacquer and from a suspension of sub-micron poly(ε-caprolactone) particles with an oil-filled core were determined in vitro. The size, morphology and drug encapsulation efficiency of the particles, and the in vitro release of CPX therefrom, were characterized. Drug permeation and uptake experiments were performed on human nail samples that were either intact, or microneedle-porated, or pre-treated with 10% N-acetyl cysteine and then microneedle-porated.

Results: Spherical particles, from which sustained release of CPX was achieved, were produced with an average diameter of 317 nm. After 14 days of application, CPX was taken up into all nails treated with the particle suspension; however, complete permeation of drug was only achieved across fully hydrated nails that had been microneedle-porated following pre-treatment with N-acetyl cysteine. The lacquer, in which the drug concentration was 25-fold higher than that in the particles, delivered significantly more CPX into the nails but, again, the drug did not penetrate across the entire structure without the {microneedle + N-acetyl cysteine} pre-treatment. Significant drug crystallization on the nail surface was also observed post-application of the lacquer.

Conclusions: Although the delivery of CPX into the nail from sub-micron particle suspension was less than that achieved by a marketed commercial lacquer formulation, the amount of drug taken up nonetheless exceeded its minimum inhibitory concentration against pathogens, such as T. rubrum and T. mentagrophytes, which cause onychomycosis. Further work is clearly required, however, to increase drug loading into the particle formulations and to optimize methods to enhance drug penetration across the nail.
Chapter 6

6.1 Introduction

Onychomycosis is the most common nail disorder and is currently treated with orally and/or topically delivered antifungal drugs. Neither approach is particularly successful: oral administration requires long courses of treatment with attendant problems of systemic side effects and drug-drug interactions, while topical delivery is significantly impeded by poor drug penetration across the highly keratinized structure of the nail. Indeed, the clinical outcome of topical treatments with medicated nail lacquers is typically so unsatisfactory that topical monotherapy is only recommended for the treatment of mild infections (1).

The use of nano- and sub-micron polymeric particle-based delivery systems for the topical treatment of skin diseases has been the subject of considerable recent research (2). One focus of the work described in this chapter is to explore the potential use of such particulate formulations in the topical treatment of nail disease. The ability of poly(ε-caprolactone) nanoparticles to sustain the release of a model ‘active’ into the nail has been demonstrated (Chapter 4-5). Here, the approach is applied to the delivery of ciclopirox (CPX) (Figure 6.1), a common antifungal drug, into and across the nail. The use of microneedle, and {microneedle + chemical enhancer} pre-treatments to enhance drug uptake and permeation were also investigated. The performance of the particle suspension was compared to that of a commercially available nail lacquer.

![Figure 6.1: Structure of ciclopirox (CPX). Molecule weight = 207.3 Da.](image)

6.2 Materials and methods

6.2.1 Chemicals

Preformed polymer PCL (MW: 10,000), poloxamer 188 (Kolliphor® P188), oleic acid and N-acetyl-cysteine were purchased from Sigma-Aldrich Co. (Gillingham, UK). Ciclopirox olamine (MW 268.4 Da) was obtained from Fagron Iberica (Barcelona, Spain). Ciclopoli® Nagellack (Taurus Pharma, Bad Homburg, Germany) was purchased from a local Germany pharmacy.
6.2.2 Nail sample preparation

Human fingernail clippings (at least 8 mm in length) were obtained from healthy volunteers who gave informed consent. Ethical approval was granted by the Research Ethics Approval Committee for Health (REACH; EP 11/12 115) of the University of Bath (Forms in Appendix 1). Nails were maintained frozen (-20°C) until use. Prior to the experiments, the nail was soaked in deionized water for 30 minutes to restore some flexibility. Microporation was performed using a commercially available dermaroller (Infinitive Beauty®, Birmingham, UK) with titanium needles (250 µm in length) that was applied to the dorsal side of the hydrated nail by rolling it back and forth a total of 5 times. Chemical pre-treatment involved soaking the entire nail in an aqueous (10% w/v) N-acetyl cysteine solution for 24 hours.

6.2.3 Sub-micron particle preparation

100 mg of ciclopirox olamine, dissolved in 1 mL of oleic acid, was introduced into an acetone solution of PCL (250 mg in 20 mL). The resulting mixture was injected drop-wise into an aqueous phase (100 mL, comprising 0.5% w/v Kolliphor® 188 in 100 mM sodium acetate pH 5 buffer solution) with vigorous stirring (1000 rpm) to form a milky suspension. The acetone was then removed under reduced pressure and the CPX-particle suspension was filtered (0.45 µm nylon filters, Whatman, Maidstone, UK) to remove any large precipitates.

6.2.4 Sub-micron particle characterization

Particle size and polydispersity index were measured (n=3) using dynamic light scattering (ZetaSizer Nano S, Malvern, UK), following a 100-fold dilution of the suspension.

Particle morphology was imaged using a field emission scanning electron microscope (FESEM) (6301F, JEOL Ltd., Tokyo, Japan) equipped with a cryo transfer system (Alto 1500CT, JEOL) which comprises a cryo (preparation) chamber and a cold stage. The particle suspension was pipetted onto a filter paper and placed in the cryo-specimen holder. The sample was then snap-frozen by plunging into liquid nitrogen slush and transferred under vacuum into the specimen process stage. The specimen surface was cleaved with a cold knife integrated within the chamber and then lightly etched (ice sublimation) in a controlled manner. Finally, the sample was loaded onto the cold stage in the microscope for imaging.
The amount of CPX entrapped in the particles was calculated from the difference between the quantity found after their complete dissolution in acetonitrile:water (50:50) ($Q_{tot}$), and that in the aqueous supernatant ($Q_{sup}$) after ultrafiltration-centrifugation of the suspension at 6000 rpm for 30 minutes (MWCO 3000, Millipore Corp., Watford, UK). All experiments were performed in triplicate. The entrapment efficiency (%) was given by: $\{100 \times [(Q_{tot} - Q_{sup}) / Q_{input}]\}$, where $Q_{input}$ represents the initial drug input.

### 6.2.5 In vitro CPX release

The release of CPX from (a) the particle suspension, (b) a solution of CPX in water at the equivalent concentration to that in the suspension, and (c) Ciclopoli® nail lacquer, was determined under ‘closed’ conditions (Figure 6.2). 300 µL of formulation was placed in an Eppendorf tube, which was covered with a dialysis membrane (Spectra/Pore® regenerated cellulose membrane 1,000 MWCO; Spectrum Laboratories, USA) and closed with a pierced cap, to provide a diffusion area of 0.78 cm². The inverted Eppendorf was placed inside a centrifuge tube filled with the receptor solution, 20 mL of pH 7.4 phosphate buffer saline (PBS). The system was constantly stirred and maintained at 32°C. The receptor solution was sampled at fixed time intervals and assayed for CPX content. For the lacquer, CPX release was also measured in the same way but under ‘open’ conditions (Figure 6.2), where the donor compartment was exposed to ambient conditions.

![Figure 6.2: Schematic diagram showing the setup for CPX release studies in ‘enclosed’ (left) and ‘open’ (right) conditions.](image)

### 6.2.6 Nail permeation of CPX

Nail permeation experiments were performed using a diffusion cell set-up (Figure 6.3), which was deliberately designed with a small receptor volume to maximize, for
analytical purposes, the drug concentration in the withdrawn samples. The nail (with or without pre-treatment) was mounted in the specialized adaptor (PermeGear Inc., Bethlehem, PA, USA, diffusion area = 0.2 cm²) with the dorsal surface facing the donor chamber, while the ventral side was hydrated by 500 µL of pH 7.4 PBS, containing 30 mg/L sodium azide to prevent microbial growth. 300 µL of either the particle suspension or the lacquer was introduced into the donor compartment, which is either exposed to air or enclosed. The entire set-up was placed in an incubator at 32°C and gently shaken throughout the experiment. 300 µL of the receptor medium was sampled every 48 hours and replaced with fresh receptor medium. The permeation study lasted for 14 days.

Figure 6.3: Schematic diagram showing the setup for nail permeation studies. (a) Cross-sectional view of the assembly. The donor compartment is either (b) exposed to air or (c) enclosed.

6.2.7 Recovery of CPX from nails

At the end of the permeation study, the nail was removed from the diffusion cell and thoroughly washed with deionized water. The central region of the nail, which was exposed to the drug formulation, was separated from the peripheral areas (Figure 6.4), weighed and then reduced to small fragments that were placed in an Eppendorf tube with 1 mL of an ethanol-water (70:30) mixture. The Eppendorf was then tightly sealed with Parafilm® and drug extraction from the nail was carried out by shaking the tube at room temperature for 7 days. Subsequently, the solution was passed through a Cronus® 0.45 µm syringe filter and assayed for CPX content. This method of extraction was
validated by spiking a nail with a solution containing a known quantity of CPX and leaving it to dry for 4 hours. The excellent recovery of 93 ± 6% (n=3) implies that CPX does not bind irreversibly to any significant extent to the nail. Analysis of the extraction medium following contact with nail samples in the absence of any CPX confirmed that there was no interference with the assay due to extraction of endogenous compounds.

Figure 6.4: Area of nail exposed to CPX formulations (circled in red) and the peripheral area of the sample.

6.2.8 High performance liquid chromatography (HPLC)

CPX was quantified using HPLC (Dionex Sunnyvale, CA, USA) with UV detection at 305 nm. A 5 µm Purospher® STAR RP-18 endcapped 150 x 4.6 mm column (Merck KGaA, Darmstadt, Germany) was employed. The mobile phase was a 68:32 v/v mixture of acetonitrile and an aqueous solution containing 20 mM orthophosphoric acid and 0.05 mM EDTA disodium salt, flowing at 1 mL/min. The injection volume was 40 µL and the system was maintained at 25° C. The retention time of CPX was 3.6 minutes. The limits of detection and quantification were calculated as 0.21 and 0.63 µg/mL, respectively.

6.2.9 Data analysis

Data analysis was performed with Graph Pad Prism version 5.00 (Graph Pad Software, San Diego, California USA). The apparent steady-state fluxes of CPX across the nail were estimated from the slope of the linear part of the cumulative permeation profiles. The lag time was estimated by extrapolation to the time-axis in the usual way. The amounts of CPX extracted from the nails treated with the lacquer or the particle suspension, and pre-treated as previously described, were compared using a two-way analysis of variance on the factors ‘formulation’ and ‘pre-treatments’, followed by a
Bonferroni post-test. The quantities of CPX extracted from the particle suspension-treated nails, either microneedle-porated or exposed to N-acetyl cysteine and then porated, were compared using unpaired t-tests. The level of statistical significance was set to \( p \leq 0.05 \) in all cases.

6.3 Results and discussion

6.3.1 Sub-micron particle characterization

Although CPX olamine salt has reasonably good water solubility (32.8 mg/mL) (3), drug-loaded sub-micron particles were prepared using the solvent displacement (nanoprecipitation) method (4), which is more typically used to entrap poorly water-soluble compounds. It has been proposed that incorporation of a liquid core in which drug solubility is relatively high can increase its entrapment in the polymeric particles (5). Equally, modification of the pH of the continuous aqueous phase to lower drug solubility therein can also promote entrapment into the particles (6-8). Here, both strategies were adopted. First, oleic acid (OA), a naturally occurring fatty acid and a commonly used skin penetration enhancer, is a good solvent for CPX (> 200 mg/mL) (9), and was used therefore as the oil-core, in which to solubilize the drug in the sub-micron particles. Second, as CPX is a weak acid (pKa ~8.07), its partitioning into the particles during their precipitation was promoted by using a pH 5.0 acetate buffer as the aqueous phase. The predominantly unionized nature of CPX at this pH, and its associated lower solubility (10), diminished its tendency to migrate into the continuous phase, and to remain within the particles, both during preparation and upon subsequent storage.

The average particle diameter was 317 ± 8 nm with a reasonably small polydispersity index of 0.172 ± 0.025 (Figure 6.5); the individual particles were spherical (Figure 6.6). The concentration of CPX in the suspension was 3.08 ± 0.01 mg/mL. The encapsulation efficiency was 58.7 ± 0.3 % and a small amount of free, unencapsulated CPX (2.98 ± 0.2%) was detected in the suspension.
Figure 6.5: Size (diameter) distribution profiles of CPX-loaded PCL particles (n=3).

Figure 6.6: SEM images of CPX-loaded PCL particles. Scale bar = 1 µm.

6.3.2 In vitro release studies

The release kinetics of CPX from (a) a solution in water, (b) the particle suspension and (c) Ciclopoli® nail lacquer are shown in Figure 6.7. From the aqueous solution, 33% was released within 30 minutes, and 100% by 6 hours. Drug release from the particles showed a classic, initial burst effect (14%) during the first ~8 hours, and about half of the drug loading had been liberated after 48 hours. It is reasonable to suggest that the early release of the drug is attributable both to diffusion of the small amount of unencapsulated drug, as well as the rapid loss of the water-soluble CPX from the external surface of the particles (6, 11, 12). The subsequent, slower release of drug was presumably determined by its diffusion from the particle oil core and through the polymeric shell.

The CPX release profiles from the particles were fitted to the Higuchi and Korsmeyer-Peppas models (Figure 6.8), which have been widely used to analyze these types of data (13). The Higuchi, ‘square root of time’ approach (14) is founded on the appropriate solution to Fick’s 2nd law of diffusion. In contrast, the Korsmeyer-Peppas interpretation
(15) examines whether the data indicate any significant deviation from Fickian behaviour. In this case, the slope of a log-log plot of cumulative release versus time provides an exponent ($\eta$) which, if $\leq 0.5$, is indicative of Fickian diffusion. Values of $\eta \geq 1$, however, suggest that drug release behaviour is more complex and that at least one parallel process, in addition to simple diffusion, is taking place. As seen in Figure 6.8, both models fit the data rather well, with $r^2$ values close to 0.97. The value of the Korsmeyer-Peppas $\eta$ was 0.69, indicative of a degree of deviation from simple diffusion.

![Graph showing cumulative release over time for different conditions](image)

Figure 6.7: Cumulative percentage of CPX payload released as a function of time from aqueous solution, particle suspension, and Ciclopoli® nail lacquer (mean ± SD, n=3). The release experiments were performed under either ‘closed’ (black lines) or ‘open’ (red line) conditions.

![Graph showing fits of Higuchi and Korsmeyer-Peppas models](image)

Figure 6.8: Fits of the (a) Higuchi, and (b) Korsmeyer-Peppas models to the profiles of CPX release from the particle suspension (mean ± SD).

The commercially available product, Ciclopoli®, is an organic nail lacquer containing 8% w/w CPX as a free acid, ethyl acetate, ethanol, cetylstearyl alcohol and the hydrophilic polymer, hydroxypropyl-chitosan. It is noteworthy that the drug amount in this formulation is ~25-fold higher than that in both the particle suspension and the
aqueous solution. For the lacquer, CPX release was evaluated under ‘closed’ and ‘open’ conditions, with the latter mimicking the air exposure that would occur with the practical use of lacquer, where the volatile solvent evaporates upon application, forming, in principle, a clear film on the nail surface. However, instead, drug crystallization was observed within 30 minutes under ‘open’ conditions (Figure 6.9) and had also occurred by 6 hours when the system was ‘closed’. Intriguingly, the release profiles for the two conditions were essentially identical (Figure 6.7), despite the different rates at which CPX crystallization was observed. The appearance of solid drug is hardly surprising, most likely due to rapid solvent evaporation and its faster penetration (relative to CPX) across the membrane. At the same time, though, water from the receptor chamber is unquestionably (and probably quickly) diffusing in the opposite direction to an extent that will at least keep a portion of the CPX in dissolved and diffusible form in the donor compartment. The end result of these concomitant processes appears to be that, by the end of the release experiment after 48 hours, the cumulative percentage of the CPX payload delivered from the lacquer is about 25% under both ‘closed’ and ‘open’ conditions.

![Figure 6.9: CPX crystals formed over time on the ‘donor’ surface of the membrane during the in vitro release test of the nail lacquer, Ciclopoli® under ‘open’ conditions.](image)

### 6.3.3 Nail uptake of drug and permeation measurements

The first experiments compared the uptake and permeation of CPX from the lacquer and the particle suspension under ‘open’ conditions into and through nails that were either (a) intact, (b) microneedle-porated, or (c) treated with N-acetyl cysteine before microneedle poration. The results are summarized in Table 6.1.

CPX delivered from Ciclopoli® only permeated through nails, which had been microneedle-porated and pre-treated with N-acetyl cysteine. Drug crystallization was observed at 1.5 hours post-application and persisted thereafter until the end of the experiment (Figure 6.10). Although the lacquer is claimed to form an invisible film upon application to the nail (16), this was refuted by the detailed microscopic examination performed.
Table 6.1: CPX uptake into and permeation through human nails under ‘open’ conditions.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ciclopoli®</th>
<th>Sub-micron particle suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPX applied (mg/mL)</td>
<td>80</td>
<td>3.07</td>
</tr>
<tr>
<td>Nail pre-treatment</td>
<td>None (Intact)</td>
<td>MNs&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nail thickness (µm)</td>
<td>320 ± 53</td>
<td>347 ± 32</td>
</tr>
<tr>
<td>CPX permeated in 14 days (µg/cm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt; LoD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>87.9 ± 50.0</td>
</tr>
<tr>
<td>CPX uptake in 14 days (µg/mg)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.89 ± 1.56</td>
<td>3.10 ± 0.72</td>
</tr>
</tbody>
</table>

<sup>a</sup>MNs = microneedles
<sup>b</sup>N-AC = N-acetyl cysteine.
<sup>c</sup>LoD = limit of detection.
<sup>d</sup>Mean ± SD; n=3 or 4.

Figure 6.10: Solid crystals formed during the nail permeation study of Ciclopoli®. Photos were taken at the times indicated along the top.

The CPX permeation profile from the lacquer across the N-acetyl cysteine pre-treated, microneedle-porated nail is shown in Figure 6.11. The apparent steady state flux and the lag time were 6.68 ± 1.75 µg d<sup>-1</sup> cm<sup>-2</sup> and 10 hours, respectively. N-acetyl cysteine is a sulphhydryl (S-H)-containing amino acid derivative that cleaves disulphide bonds in the keratin matrix, and has been reported to promote drug permeation across bovine hoof membranes (10) and human nail plates (17).
Figure 6.11: Cumulative delivery (n=3, mean + SD) of CPX from Ciclopoli® across N-acetyl cysteine pre-treated and microneedle-porated nails.

In previously published work, when the same lacquer was applied to bovine hoof slices (80-120 µm), a very short lag time (~1.3 hours) and a high CPX flux (equivalent to 153 ± 29 µg d⁻¹ cm⁻²) were reported (18). This finding is consistent with the fact that the keratin structure in bovine hoof membranes is much looser and considerably more permeable (up to 30-fold) (19, 20) than healthy human nails. Across infected human toenails, the apparent flux of CPX from Ciclopoli® within 5 days was 0.52 ± 0.20 µg d⁻¹ cm⁻² (21). It appears therefore that the pathological effect of dermatophytes on nail structure certainly increases its permeability over that of the intact, normal nail, but is less damaging than a combination of N-acetyl cysteine and microneedle pre-treatments.

Post-application of the polymer particle suspension, a transparent film formed on the nail surface after evaporation/penetration of water. While CPX was clearly taken up into the nail over 14 days (Table 6.1), it had not permeated sufficiently to be detectable in the receptor solution, even when microneedle or {chemical + microneedle} pre-treatments were used. Two-way ANOVAs were performed to compare the uptake of CPX into the nail from the two formulations as a function of the pre-treatments employed. It was found that uptake from the lacquer was significantly higher than that from the particle suspension (p < 0.05), but that, for both formulations, uptake was statistically indistinguishable (p > 0.05) between intact nails, those porated with microneedles, and those pre-treated with N-acetyl cysteine prior to microneedle poration. Given that nail density is known (1.33 g/mL) (22), it was possible to calculate the concentration ranges of CPX in the nail after treatment with the lacquer and particle formulations: 2.94-14.3 mg/mL and 0.43-1.97 mg/mL, respectively. Importantly, these values are well above the reported minimum inhibitory concentration (MIC) range (0.015-0.125 µg/mL) (23) of CPX against dermatophytes causing onychomycosis, including T. rubrum and T. mentagrophytes.
As CPX permeation from the particle formulation could not be quantified under ‘open’ conditions, the experiment was repeated under occlusion maintaining, thereby, the nail in a fully hydrated condition. The nails were either (a) microneedle-porated, or (b) treated with N-acetyl cysteine before microneedle poration. The results are summarized in Table 6.2.

Table 6.2: CPX uptake into and permeation through human nails under ‘closed’ conditions.

<table>
<thead>
<tr>
<th>Nail pre-treatment</th>
<th>Formulation</th>
<th>Sub-micron particle suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nail thickness (μm)</td>
<td>MNs⁵</td>
<td>N-AC⁶ + MNs⁵</td>
</tr>
<tr>
<td>CPX permeated in 14 days (μg/cm²)⁷</td>
<td>&lt; LoD⁷</td>
<td>10.2 ± 5.90</td>
</tr>
<tr>
<td>CPX uptake in 14 days (μg/mg)⁸</td>
<td>0.56 ± 0.24</td>
<td>0.88 ± 0.13</td>
</tr>
</tbody>
</table>

⁵MNs = microneedles  
⁶N-AC = N-acetyl cysteine.  
⁷LoD = limit of detection.  
⁸Mean ± SD; n=3 or 4.

Once again, CPX uptake into the nail in 14 days was not influenced by the pre-treatment performed. While the pre-application of microneedles was not enough to allow CPX delivery across the nail, the immersion in N-acetyl cysteine prior to physical poration did promote drug permeation, with an apparent steady state flux of 1.32 ± 0.51 μg d⁻¹ cm⁻² and a lag time of ~6 days (Figure 6.12). Evidently, the additional impact of fully hydrating the nail (an approach known to increase its porosity (24, 25)) has enabled CPX to completely permeate the barrier. Although the flux from the particle suspension is significantly lower (~5-fold) than that from the lacquer, it is again worth noting that the loading in the former is substantially (by a factor of about 25) less than that in the commercial formulation.

Figure 6.12: Cumulative delivery (n=3, mean + SD) of CPX from the polymer particle suspension across N-acetyl cysteine pre-treated and microneedle-porated nails.
Presently, drug permeation across the highly keratinized structure of the nail remains a daunting challenge. The currently available, medicated nail lacquers suffer from rapid drug precipitation shortly post-application due to the evaporation of volatile solvent, rendering a large fraction of the applied active unavailable for subsequent partitioning into and diffusion across the barrier. The results described here demonstrate that small polymer particles, which encapsulate the drug, may be able to maintain a reservoir from which the release of the compound could be sustained over an extended period of time without the risk of crystallization. Clearly, there is room for further optimization and development of such formulations for topical drug delivery to the nail; not only to increase the applied ‘payload’ of the active, but also to take advantage of combined enhancement strategies (as illustrated here) to achieve a maximally useful outcome.

### 6.4 Conclusions
The release and nail permeation of ciclopirox (CPX) from a commercially available lacquer and from a suspension of sub-micron poly(ε-caprolactone) particles with an oil-filled core have been assessed. Experiments have been carried out on human nail clippings that were either intact, or microneedle-porated, or pre-treated with 10% N-acetyl cysteine and then microneedle-porated. CPX was taken up into all nails treated with the particle suspension; however, complete permeation of drug was only achieved across fully hydrated nails that had been microneedle-porated following pre-treatment with N-acetyl cysteine. Even though CPX delivery into the nail from the sub-micron particle suspension was less than that achieved by the lacquer formulation, the amount of drug taken up substantially exceeded its minimum inhibitory concentration against the primary pathogens which cause onychomycosis.
References

11. Niwa T, Takeuchi H, Hino T, Kunou N, Kawashima Y. Preparations of biodegradable nanospheres of water-soluble and insoluble drugs with D,L-


Chapter 7: Pluronic F127-based thermogelling formulations for topical nail delivery

Overview

Purpose: To investigate the potential use of Pluronic F127 (PF-127)-based hydrogels to deliver drugs to the nail plate by examining their in situ gelling behaviour in healthy volunteers and by assessing their ability to release model compounds in vitro and to deliver fluorescent markers into the nail.

Methods: Hydrogels with several concentrations of PF-127 were prepared and their gelling temperatures were determined. The fluorescent markers sodium fluorescein (SF) and Nile red (NR) were used as models for hydrophilic and lipophilic actives, respectively. The two markers were incorporated into the gels and their effect on the gelling temperature and their in vitro release profile was characterized. Next, the penetration of dyes into human nail clippings under two experimental conditions was assessed by laser scanning confocal microscopy (LSCM). The first experimental condition involved a continuous occluded, 7-day application and the second, a discontinuous un-occluded, 7-day application consisting of daily cycles of an 8-hour application followed by a washing step and a 16-hour period without formulation. The latter mimicked a potential practical use (night application only) of the formulations.

Results: Gels with 20%, 25% and 30% w/v of PF-127 were selected for their gelling properties and further investigated. As expected, the presence of PF-127 slowed the in vitro release of the model compounds with respect to the free solution. The three PF-127 concentrations delivered the markers into the nail plate at similar depths. Typically, the two markers penetrated 30% or less of the nail thickness during the 7-day study and as anticipated, the continuous, occlusive approach led to greater penetration than the discontinuous, un-occluded approach. The confocal images suggested that SF and NR penetrated the nail plate via the transcellular and intercellular pathways, respectively. Finally, the gelling properties were confirmed in vivo and the feedback from the volunteers suggested that the formulations would be well accepted by users.

Conclusions: Thermogelling PF-127-based formulations were easy to apply and to prepare. The aqueous based formulations provided continuous release of the markers for at least 6 hours and deliver them to the nail plate. Further characterization of the formulations incorporating antifungal drugs is warranted.
Chapter 7

7.1 Introduction

Onychomycosis is a common, persistent nail disorder which is responsible for ~ 50% of all nail diseases. Antifungal drugs can be taken orally to treat the infected nail and, through the distribution process, a small fraction of the dose reaches the nail bed and is slowly incorporated into the nail plate. While reasonably effective, oral therapy is limited by potential side effects and drug interactions. Another intervention is nail avulsion or surgical removal of the infected nail plate which is an extremely traumatic procedure (1, 2). Local treatments can circumvent these problems by targeting the drug to the infected area whilst minimizing systemic exposure. For that reason, several medicated nail lacquers are available in the market (see Table 1.2 in chapter 1) for the treatment of onychomycosis. Nail lacquers are applied as liquids using a brush and form an adhesive film on the nail surface upon application. The adhered film has a longer residence time on the nail plate than gels, creams and ointments and ideally, the drug in the film partitions into and then diffuses across the nail plate into the nail bed to reach the concentration required to fight the infection. In practice though, the efficacy of medicated nail lacquers is limited (3) so they are only used for superficial infections or in combination with oral drugs (4). One cause for the reduced efficacy is the thickness and the highly keratinized structure of the nail plate that limits drug permeation so the amount of drug reaching the nail bed is insufficient to treat the infection effectively (2, 5). On the other side, poor formulation is another contributing factor. Most lacquers contain high levels of organic solvents that evaporate rapidly upon application. Unfortunately, these solvents are often required to solubilize the drug and, after a transient state of super-saturation, drug crystallization occurs. The active therefore, becomes unavailable for any further partitioning into the nail and its delivery stops.

In summary, there is a need to improve and optimize nail dosage forms and particularly to ensure that nail formulations keep the active available for delivery for extended periods of time. The previous chapters have explored the use of nanoparticles as potential reservoirs for antifungal drugs; this chapter will explore the potential of organic solvent-free, thermoresponsive, in situ gelling hydrogels, relatively simpler systems, to deliver drugs to the nail plate. Recent work with ciclopirox olamine showed that an aqueous nail lacquer containing 20% PF-127, cyclodextrins and penetration enhancers provided better nail and hoof penetration of the actives than a marketed organic formulation (6).
This study will investigate further the use of Pluronic F-127 (PF-127)-based systems for topical nail delivery. PF-127 is one in the series of polyethylene oxide-polypropylene oxide-polyethylene oxide (PEO-PPO-PEO) block copolymers, the members of which share the general formula:

\[
\text{H(O-CH}_2\text{-CH}_2)_a\text{(O-CH-CH}_2)_b\text{(O-CH}_2\text{-CH}_2)_a\text{-OH}
\]

\[
\text{CH}_3
\]

PF-127 has a nominal molecular weight of 12,500 daltons and contains 70-79% the hydrophilic portion, PEO (7, 8). PF-127 is commercially available, odourless, and water-soluble. It is considered safe and has been approved by FDA (Food and Drug Administration) for use as food additives and pharmaceutical ingredients (9). Moderately concentrated (above 20%) aqueous solutions of PF-127 exhibit the interesting phenomenon of reversible thermal gelation. That is, they present themselves as free-flowing liquids at refrigerated temperatures and as gels at standard room temperature (10). This behaviour is related to the micellar structure of PF-127 over the temperature range of 10-40° C. At low temperature, the polymers are fully dissolved and transform into a spherical micellar phase when the critical micellization temperature (CMT) is reached. The concentration of micelles further increases as the temperature increases and once the gelation temperature (T_{gel}) is reached, the volume density of micelles is sufficiently high that the polymers are ‘locked’ into a crystalline cubic structure of hard spheres, forming a solid gel (8). It is believed that gelation occurs as result of the dehydration of the hydrophilic PEO portion, leading to chain friction and entanglement that favours hydrophobic interactions of the PPO domains (7). This distinctive thermoreversible gelling behaviour of PF-127 has been widely exploited for the formulation of topical drug delivery systems (11-13).

The present study aims to further characterize PF-127-based vehicles as topical nail drug delivery systems and primarily whether they meet some essential criteria. First, that the gelling behaviour of PF-127 solutions in vivo allows the quick formation of a layer on the nail plate and second, that the actives incorporated in the formulations do not crystallize upon formation of this layer and remain available to partition into the nail plate for extended periods of time. Additionally, given the water-soluble properties of these formulations, a night application of the lacquer is envisaged and therefore delivery of the active would be intermittent rather than continuous. To answer these questions
sodium fluorescein (SF) and Nile red (NR) were incorporated into the vehicles as models for hydrophilic and lipophilic actives and their in vitro release profiles were characterized. Next, the nail penetration of the two markers incorporated in PF-127 formulations and applied either continuously or discontinuously was studied using laser scanning confocal microscopy (LSCM). A panel of healthy volunteers was involved to assess the acceptability of these formulations and their gelling behaviour in vivo.

7.2 Materials and methods

7.2.1 Chemicals
Pluronic F-127 (PF-127), sodium fluorescein (SF), Nile red (NR) and polysorbate 80 (Tween 80) were purchased from Sigma-Aldrich Co. (Gillingham, UK). Polyethylene glycol 400 (PEG-400) was obtained from Acros Organic (New Jersey, USA).

7.2.2 Nail sample preparation
Human fingernail clippings (at least 8 mm in length) were donated by healthy volunteers who gave informed consent. Ethical approval was granted by the Research Ethics Approval Committee for Health (REACH; EP 11/12 115) (Forms in Appendix 1) of the University of Bath. The nail clippings were maintained frozen (-20°C) until use. Prior to the experiments, the nail’s thickness was measured using a point micrometer (Point Anvil Micrometer, Mitutoyo, Andover, UK). The nail samples were then soaked in deionized water for 30 minutes to restore some flexibility before the experiments.

7.2.3 Volunteers
18 healthy volunteers (13 males and 5 females, age 18-53 years) not suffering from any skin or nail diseases and allergies and who had not used any cosmetic nail treatment recently were recruited for the in vivo study. Informed consent was obtained from all participants. Ethical approval for the in vivo study was granted by the Research Ethics Approval Committee for Health (REACH; EP/13/14 27) (Forms in Appendix 2) of the University of Bath.

7.2.4 PF-127 gels preparation
PF-127 gels were prepared using a ‘cold’ method. PF-127 (10, 15, 20, 25, 30 and 35% w/v) was added to cold, stirring deionized water and kept at 4°C until complete dissolution.
SF-PF-127 gels were prepared by dissolving the necessary amount of SF in cold PF-127 solutions so the final amount of the marker was 0.1% w/v.

NR-PF-127 gels were prepared by adding NR dissolved in PEG-400 to cold PF-127 solutions so the final concentration of NR and PEG-400 were 0.002% w/v and 10% v/v, respectively. The mixture was then kept at 4°C with gentle stirring until a homogeneous solution was formed.

7.2.5 Determination of gelation temperature ($T_{gel}$)

$T_{gel}$ was determined as the lowest temperature at which a gel can hold its weight in an inverted vial (Figure 7.1). 1 mL of the cold gel solutions were introduced into an Eppendorf tube that was placed in a water bath at ~10°C temperature. The temperature of the water bath was increased by 1°C every two minutes until the transition to solid gel occurred.

Figure 7.1: Determination of $T_{gel}$ by the ‘inverted tube’ method.

7.2.6 In vitro release studies

The in vitro release of SF and NR from PF-127 gels and control aqueous solutions was characterized using the system shown in Figure 7.2. Briefly, 300 µL of the cold PF-127 formulations were introduced into an Eppendorf tube with a pierced cap (0.20 cm²) and sealed with a diffusion membrane. Spectra/Pore® regenerated cellulose dialysis membrane (1,000 MWCO; Spectrum Laboratories, USA) and silicone membrane (no. 7-4107, 75 µm thick; Dow-Corning, Coventry, UK) were used for SF and NR experiments, respectively. The Eppendorf tube was inverted and incubated at 32°C for 10 minutes so the formulation gelled on top of the diffusion membrane. Next, the inverted tube was placed inside a centrifuge tube filled with the receptor solution and
also kept at 32°C. The receptor media were 20 mL phosphate buffer solution (PBS) pH 7.4 for SF and 10 mL of Tween 80 (5% w/v) in pH 7.4 PBS for NR and were continuously stirred. Samples of the receptor were taken at fixed time intervals and replaced with fresh solution. The concentration of SF and NR in the samples was quantified by HPLC. Control experiments were conducted in the same way and used 0.1% w/v SF in water and 0.002% w/v NR in PBS containing 5% w/v Tween 80 and 10% v/v PEG-400 as donor solutions; both dyes were at the equivalent concentrations to the PF-127 formulations.

Figure 7.2: Experimental setup used for in vitro release studies.

7.2.7 High performance liquid chromatography (HPLC) analysis
SF and NR were quantified using HPLC-fluorescence detector (Dionex, Sunnyvale, CA, USA). A HiQ sil C18W 250x4.6 mm column (KYA Tech, Tokyo, Japan) was employed and the system was maintained at 25°C. Table 7.1 shows the details of the quantification methods.
Table 7.1: Quantification methods of SF and NR using HPLC.

<table>
<thead>
<tr>
<th></th>
<th>Sodium fluorescein (SF)</th>
<th>Nile red (NR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation wavelength (nm)</td>
<td>488</td>
<td>559</td>
</tr>
<tr>
<td>Emission wavelength (nm)</td>
<td>515</td>
<td>630</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Methanol:0.05 M potassium dihydrogen phosphate buffer pH 7.4 (52:48)</td>
<td>Methanol: water (95:5)</td>
</tr>
<tr>
<td>Flow rate (mL/min)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>LoD (ng/mL)</td>
<td>3.6</td>
<td>12.7</td>
</tr>
<tr>
<td>LoQ (ng/mL)</td>
<td>11.0</td>
<td>38.6</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>3.3</td>
<td>6.5</td>
</tr>
</tbody>
</table>

LoD = limit of detection.
LoQ = limit of quantification.

7.2.8 *In vitro* nail permeation studies

Nail permeation experiments were performed using vertical Franz diffusion cells including nail adaptors (PermeGear Inc., Bethlehem, PA, USA, diffusion area = 0.2 cm²). The dorsal surface of the nails faced the top, donor chamber. The ventral side of the nails was kept in contact with the pH 7.4 PBS in the receptor chamber (7.5 mL) throughout the experiments. The whole set up was maintained at 32° C. Two types of experiments were performed:

Continuous application experiments: In this case, 300 µL of the gel solution were applied and left on the nail surface for 7 consecutive days. Both the donor and receptor chambers were covered with Parafilm® to create an occluded environment.

Discontinuous application experiments: These aimed to investigate the penetration of the markers into the nail plate in conditions mimicking a more practical scenario. That is, the overnight application of the formulations followed by their removal through hand washing in the morning. To simulate the overnight application, 20 µL of PF-127 solutions were applied onto the nail plate and left for 8 hours. The formulations formed a thin layer of gel on dorsal surface and were exposed to ambient air. The receptor compartment was occluded throughout the experiments and the ensemble was kept at 32° C. After this 8 hours dosing period, the gel was removed with deionized water and the nail surface was completely dried with cotton wool bud. The formulation-free period lasted 16 hours and was followed by a new 8-hour dosing step. This cycle was repeated for 7 days.

At the end of the 7-day continuous and discontinuous experiments, the diffusion cell was dismantled and the formulation at the surface of the nail was washed off using deionized water and dried with absorbent tissue. The treated area of the nail was then immediately examined by LSCM.
7.2.9 LSCM imaging

An LSCM 510Meta inverted laser scanning microscope (Carl Zeiss, Jena, Germany) was employed to image the penetration of the two markers into the nail plate. An argon laser (488 nm) and a HeNe laser (543 nm) were used to excite SF and NR, and the corresponding emission signals were collected using a BP 505-530 (green) and LP 560 (red) filters, respectively. Dorsal and transversal confocal images were captured to localize the fluorophores on and within the nail. Dorsal images were acquired using an EC Plan-Neo 40x/1.3 oil objective. Optical section x-y planar images were obtained every 2 µm in the z-direction. To obtain transversal images, the nail clippings were sectioned into thin slices, which were then glued on to a microscope slide with the cross-section oriented towards the objective. A Plan-Apochromat 10x/0.45 M27 air objective was employed to obtain a direct transversal view of the nail. Reflectance and/or optical images were captured simultaneously and all images were taken at a few microns below the cut surface to avoid artefacts caused by the sectioning.

The penetration depth (T) of the fluorescent markers was estimated from the transversal images. The normalized penetration depth (%T) was determined by normalizing each T value with respect to the thickness of the corresponding nail measured in simultaneously taken images. To take into account both inter- and intra-individual variability, three replicate experiments were performed for each formulation and each nail sample was imaged at 3 different regions. All images were processed using ImageJ software (version 1.48v, National Institutes of Health, USA).

7.2.10 Data analysis and statistics

Data analysis was performed using GraphPad Prism version 5.00 (Graph Pad Software, San Diego, California, USA). Non-parametric Kruskal-Wallis analysis followed by a Dunn’s post-test was used to compare the T and %T values obtained for different nails and formulations. The overall difference between the continuous and discontinuous application was examined by two-way analysis of variance on the factors ‘treatment approach’ and ‘PF-127 concentrations’ followed by a Bonferroni post-test. The level of statistical significance was set to p ≤ 0.05.

7.2.11 In vivo application

The 18 healthy participants were randomly assigned to 3 equal size groups. Placebo (i.e., without fluorescent markers) formulations containing 20%, 25% and 30% of PF-
127 in distilled water were used for the study. Two different formulations were tested in each group, so each formulation was tested on 12 individuals. 20 µL of the cold formulation were applied to the nail plate using a small paintbrush. Each formulation was applied to the five fingernails of one hand. A maximum of 10 minutes was allowed for the formation of a thin coating layer on the nail surface. The layer was visually inspected for its physical appearance (uniformity, presence of bubbles). The participants were asked to complete a questionnaire and to comment on the possible use of the formulations as medicated nail lacquers.

### 7.3 Results and discussion

#### 7.3.1 \(T_{gel}\) of PF-127 formulations

The \(T_{gel}\) of unloaded formulations was first determined (Table 7.2). According to previous work (14-16), the thermogelling behaviour appears at PF-127 concentrations equal or superior to 20%. So, as expected, the 10% and 15% aqueous solutions of PF-127 did not gel in the 10-60º C range of temperatures investigated. Once the thermogelling behaviour was acquired, the results agreed with the previously reported linear correlation between polymer concentration (20-35%) and \(T_{gel}\) (14). The increased polymer concentration leads to a higher number of micelles which occupy a larger volume, and therefore, the sol-gel transition occurs at lower temperatures (17). This trend was also observed in this work. A \(T_{gel}\) between 22-30º C would be suitable for \textit{in situ} gelling upon topical nail application and therefore, the formulations with 20%, 25% and 30% of the polymer were selected for further \textit{in vivo} placebo testing and for \textit{in vitro} studies incorporating the two fluorescent markers. The formulation with 35% of PF-127 (\(T_{gel} = 18º C\)) was eliminated as the high viscosity made it difficult to apply evenly.
Chapter 7

Table 7.2: $T_{gel}$ (°C) of unloaded and loaded PF-127 formulations. NG = no-gel formed.

<table>
<thead>
<tr>
<th>PF-127 (% w/v)</th>
<th>Unloaded</th>
<th>+ SF</th>
<th>+ NR+ PEG-400</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>NG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>NG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>25</td>
<td>26</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>30</td>
<td>22</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>35</td>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Next, the thermogelling behaviour of 20%, 25% and 30% w/v PF-127 solutions loaded with SF and NR as models for hydrophilic and hydrophobic actives, respectively, was examined. This was required as the sol-gel phase transition is sensitive to the presence of co-solutes and a change in $T_{gel}$ may occur depending on the co-solute physicochemical properties and its interactions with the polymer (14, 18, 19).

Incorporation of SF decreased the $T_{gel}$ by 2-3° C (Table 7.2). This observation could be explained by a ‘salting-out’ effect or namely, the competition for water between the salt (SF) and the polymer leading to a reduced ability of solvent (water) for both PPO and PEO chains and an increased effective concentration of polymer in the system. This, therefore, favours the formation of micelles at lower temperature (20, 21).

NR-loaded formulations exhibited a further reduction in $T_{gel}$ (Table 7.2). These formulations included 10% PEG-400 for solubilization purposes so the decrease in $T_{gel}$ could be resulted from co-solvent effects by PEG as well as from the presence of NR. It has been reported that PEG-400 lowers the critical micelle concentration (CMC) of PF-127 and therefore, promotes polymer transition into a micellar phase at lower temperatures (22). It is also believed that high viscosity conferred by the PEG-400 provides a stabilizing effect that strengthens the gel texture at lower temperatures (23).

While PEG can form mixed micelles with PF-127 and preclude the process of micellar association of the latter, this effect is mostly caused by PEG with longer chain length (Mn = 2000-20,000 g mol$^{-1}$) (24-26) and is unlikely to occur with PEG-400 (27).

Finally, the affinity of NR for both the hydrophobic PF-127 chains and the polyoxyethylene chains of the PEG-400, may dehydrate the polymers and promote the entanglement of adjacent micelles, enabling gelation at lower temperatures (25).

7.3.2 **In vitro release of SF and NR from PF-127 gels**

The release profile of SF and NR from 20%, 25% and 30% PF-127 gels and from control solutions was investigated. All the PF-127 formulations maintained a gel
appearance throughout the experiment. Figure 7.3 shows that the presence of PF-127 slowed down the release of both markers; the gels with 25% and 30% polymer behaved very similarly. It has been suggested that the number and size of micelles within the gel structure increases, and the availability of aqueous channels decreases, with polymer concentration so the pathway for solute diffusion becomes more tortuous (16). The plots of amount released (per unit area) against the square root of time (Figure 7.4) show good linearity ($r^2 \geq 0.95$). In principle, the Higuchi diffusion model could be used to fit the release data (28) (Eq. 7.1):

$$\frac{Q}{A} = 2 \cdot C_0 \cdot \left(\frac{D_{\text{app}}}{\pi}\right)^{1/2}$$  \quad (Eq. 7.1)

where $Q$ is the amount of solute released per unit area ($A$); $C_0$ is the initial concentration of solute in the vehicle; $t$ is time; and $D_{\text{app}}$ is the apparent diffusion coefficient which can be calculated from the gradient of the Higuchi plot. $D_{\text{app}}$ decayed exponentially (Figure 7.5) with the polymer concentration of PF-127. This agrees with previous work (16), suggesting that substantial chain interactions occur at high PF-127 concentrations ($\geq 25\%$) and that further increments in polymer content would have little effect in hindering drug diffusion. However, other explanations are possible at least in the case of SF experiments performed with dialysis membrane. For example, water diffusion from the receptor into the gel could lower the polymer concentration with respect to the original donor formulations.

![Figure 7.3: Cumulative release (mean + SD; n=3) of (a) SF and (b) NR from PF-127 formulations and control solutions.](image)
Figure 7.4: Cumulative release (mean ± SD; n=3) of (a) SF and (b) NR versus the square root of time accordingly to the Higuchi model.

Figure 7.5: Effect of concentration of PF-127 on the $D_{app}$ (mean ± SD; n=3) estimated assuming the Higuchi diffusion model for (a) SF and (b) NR.

7.3.3 LSCM imaging
The penetration of SF and NR from PF-127 gels into nail clippings following continuous and discontinuous applications was imaged by LSCM. All the PF-127 formulations maintained a gel appearance throughout the experiment. Nail penetration can be very variable (29) so several LCSM images were taken to estimate the depth of penetration with better confidence. Despite being laborious, this procedure allowed estimation of both the inter- and intra-nail variability and better comparison of the formulations tested. Only a representative selection of the images taken is presented in this chapter; the complete set of images is presented in Appendix 3.

Dorsal images captured along the z-direction provided useful mechanistic information into the penetration pathways of the markers but might have underestimated the penetration depth due to signal loss (by light absorption and scattering) with increasing imaging depth; sample thickness also limited imaging beyond the free working distance of the objective. To avoid this, the depth of fluorescence (T) inside the nail was determined using transversal images, which also provided information about %T or penetration depth relative to the entire nail thickness.
7.3.3.1 SF experiments

Table 7.3 shows information about the nail clippings used for SF experiments. The 18 nail clippings were donated by 8 female healthy volunteers. Each formulation was tested on 3 nails donated from different volunteers.

Table 7.3: Source (volunteer code, hand and finger) and thickness of the nail clippings used in SF experiments.

<table>
<thead>
<tr>
<th>Experiment code</th>
<th>PF-127 concentration (%)</th>
<th>Nail code</th>
<th>Nail thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF.Continuous</td>
<td></td>
<td>20</td>
<td>19LF1 490</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1LF3 270</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16LF4 290</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>19RF1a 500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16RF4 350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2RF5 270</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>19RF1b 540</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1LF2 350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3RF5 300</td>
</tr>
<tr>
<td>SF.Discontinuous</td>
<td></td>
<td>20</td>
<td>10LF3 400</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3LF1 350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12LF4a 280</td>
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<td></td>
<td></td>
<td>25</td>
<td>3LF5 410</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1RF1a 330</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>5RF5 330</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1RF1b 380</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12LF4b 290</td>
</tr>
</tbody>
</table>

The first digit of the nail code represents the participant number. L and R stand of left and right hand, respectively. F + digit indicate the finger number where F1 is the thumb. Nail clippings donated by the same volunteer in different occasions are indicated by the letters ‘a’ (first donation) and ‘b’ (second donation).

Figure 7.6 - Figure 7.8 illustrate SF penetration into the nail plate following continuous application of the 20%, 25% and 30% w/v PF-127 formulations, respectively. Penetration of SF into the nails is clearly visible in both dorsal images and transversal images; the latter showing clear intense, green fluorescence bands.
Figure 7.6: Representative confocal images illustrating SF (green) penetration into the nail (grey) following a 7-day continuous application of the PF-127 20% formulation under occlusive conditions. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.7: Representative confocal images illustrating SF (green) penetration into the nail (grey) following a 7-day continuous application of the PF-127 25% formulation under occlusive conditions. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.8: Representative confocal images illustrating SF (green) penetration into the nail (grey) following a 7-day continuous application of the PF-127 30% formulation under occlusive conditions. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.9 shows the depth of penetration of SF into each nail sample after 7 days of continuous and occluded exposure to the three formulations. The average intra- and inter-nail coefficients of variation were 15% and 33%, respectively, supporting the need for several measurements per nail clipping and experiment. T measurements were preferred over %T to compare the formulations and for the statistical analysis because the latter are also modified by the nail thickness. Indeed, as shown by the results, low %T values were often associated to thicker nail samples (Table 7.3). On the other hand, %T informs about the potential efficiency of the formulations to deliver the markers across the whole nail.

Overall, the 7 days continuous treatment delivered SF, on average, to the first 87–127 µm of the nail samples corresponding to 22–38% of the whole thickness. Only one sample (1LF3) treated with 20% PF-127 showed the penetration of SF beyond 50% of nail thickness. We found no significant statistical differences in the penetration depth (T) of SF when delivered by the three formulations (Figure 7.9). The statistical difference found between the relative penetrations (%T) measured for the 20% and 30% PF-127 gels was probably due to different sample thickness, as mentioned earlier.
Figure 7.9: SF penetration into nail clippings following a continuous 7-day application of PF-127 (20%, 25% and 30%) formulations under occlusive conditions. T and %T shown in the top panels and the bottom-left panel represent the average values measured for each nail (Mean + SD of 3 images) and formulation. The bottom-right panel illustrates the average T and %T for each PF-127 gel (Mean + SD of 9 images from 3 nails). The superscript letters represent statistical difference (p < 0.05) between the pairs of bars.

Next, discontinuous, un-occluded dosing experiments were performed to mimic the practical use of the formulations as normally they would be applied overnight and then removed in the morning through hand washing. In this case, smaller amounts of the solutions (20 µL) leading to formation of a thinner layer on the nail were applied and left *in situ* for 8 hours. A 16 hours period without formulation followed and the cycle repeated for 7 days. Figures 7.10 - Figure 7.12 illustrate SF penetration into the nail plate following the repeated application of the 20% (Figure 7.10), 25% (Figure 7.11) and 30% (Figure 7.12) PF-127 formulations.
Figure 7.10: Representative confocal images illustrating SF (green) penetration into the nail (grey) following a discontinuous, un-occluded 7-day application (8 h on-16 h off) of the PF-127 20% formulation. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.11: Representative confocal images illustrating SF (green) penetration into the nail (grey) following a discontinuous, un-occluded 7-day application (8 h on-16 h off) of the PF-127 25% formulation. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.12: Representative confocal images illustrating SF (green) penetration into the nail (grey) following a discontinuous, un-occluded 7-day application (8 h on-16 h off) of the PF-127 30% formulation. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
As expected, the bands of fluorescence observed in dorsal and transversal images of the nail were thinner, reflecting the shorter exposure times associated to discontinuous dosing. The absolute (T) and normalized (%T) penetration depth of SF into each nail and for each gel are summarized in Figure 7.13. Another interesting observation was the reduction in the variability observed. For example, we found no statistical significant differences among the nails treated with the same formulation.

Overall, the 7 days discontinuous treatment delivered SF consistently to the first ~50 µm of the nail corresponding to 15% of the whole thickness. No statistical differences were found among the three gels with none delivering SF deeper than 22% of the whole nail thickness. Therefore, the marker remained in the dorsal layer of the nail (30).

Indeed, it remains unclear whether drug permeation across the three nail layers (dorsal, intermediate and ventral) differs. While according to some authors the diffusion of 5-fluorouracil and flurbiprofen across the dorsal layer was the limiting factor for the drug permeation into human nail plate (31), other work reported equivalent penetration of SF into the dorsal, intermediate and ventral layers of the nail (29).
Figure 7.13: SF penetration into nail clippings following a 7-day, (8 h on-16 h off) cyclical un-occluded application of PF-127 (20%, 25% and 30%) formulations. T and %T values shown in the top panels and the bottom-left panel represent the average measured for each nail (Mean + SD of 3 images). The bottom-right panel illustrates the average T and %T for each formulation (Mean + SD of 9 images from 3 nails).

7.3.3.2 NR experiments
Table 7.4 shows relevant details of the nails used in NR experiments. Similarly, the 18 nail clippings were donated from 8 female volunteers and each condition/treatment was tested on 3 nails from different donors.
Table 7.4: Source (volunteer code, hand and finger) and thickness of the nail clippings used in NR experiments.

<table>
<thead>
<tr>
<th>Experiment code</th>
<th>PF-127 concentration (%)</th>
<th>Nail code†</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR. Continuous</td>
<td>20</td>
<td>3RF2</td>
<td>350</td>
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<td></td>
<td></td>
<td>1RF2a</td>
<td>300</td>
</tr>
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<td></td>
<td></td>
<td>2RF5</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3LF2a</td>
<td>360</td>
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<td></td>
<td>30</td>
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<td>25</td>
<td>3LF4</td>
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<td>5LF4</td>
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<td></td>
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<td>380</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12LF5</td>
<td>260</td>
</tr>
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</table>

† The first digit of the nail code represents the participant number. L and R stand for left and right hand, respectively. F + digit indicate the finger number where F1 is the thumb. Nail clippings donated by the same volunteer in different occasions are indicated by the letters ‘a’ (first donation) and ‘b’ (second donation).

Again, the continuous application approach was first adopted. Figure 7.14 - Figure 7.16 show representative confocal images of NR penetration into nail clippings following a 7-day continuous application with the three gels. As before, the penetration of the marker was well demonstrated by dorsal and transversal images; the later showing intense, red fluorescence bands within the samples.
Figure 7.14: Representative confocal images illustrating NR (red) penetration into the nail (grey) following a 7-day continuous application of the PF-127 20% formulation under occlusive conditions. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.15: Representative confocal images illustrating NR (red) penetration into the nail (grey) following a 7-day continuous application of the PF-127 25% formulation under occlusive conditions. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.16: Representative confocal images illustrating NR (red) penetration into the nail (grey) following a 7-day continuous application of the PF-127 30% formulation under occlusive conditions. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.17 shows the T and %T values estimated for each nail and formulation. Relatively smaller inter-nail variability was observed with NR and no statistical differences were found among nails within each treatment group. Overall, NR penetrated less than 100 µm or 30% into the nails (Figure 7.17) and no significant differences were observed for the depth of penetration (T) attained with the different gels. The different %T observed for the 20% and 30% gels was, again, explained by differences in nail thickness.

![NR penetration into nail clippings following a continuous 7-day application of PF-127 (20%, 25% and 30%) formulations under occlusive conditions. T and %T shown in the top panels and the bottom-left panel represent the average values measured for each nail (Mean + SD of 3 images) and formulation. The bottom-right panel illustrates the average T and %T for each PF-127 gel (Mean + SD of 9 images from 3 nails). The superscript letters represent statistical difference (p < 0.05) between the pairs of bars.](image)

The penetration of NR following discontinuous application of the same formulations was then investigated. The representative confocal images are shown in Figure 7.18 - Figure 7.20.
Figure 7.18: Representative confocal images illustrating NR (red) penetration into the nail (grey) following a discontinuous, unoccluded 7-day application (8 h on-16 h off) of the PF-127 20% formulation. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.19: Representative confocal images illustrating NR (red) penetration into the nail (grey) following a discontinuous, un-occluded 7-day application (8 h on-16 h off) of the PF-127 25% formulation. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal reconstructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.20: Representative confocal images illustrating NR (red) penetration into the nail (grey) following a discontinuous, un-occluded 7-day application (8 h on-16 h off) of the PF-127 30% formulation. Panel A shows the x-y planar images obtained by dorsal imaging every 2 µm (shown at 8 µm intervals). Panel B shows the x-z orthogonal re-constructions of dorsal images. Panel C shows the transversal cuts used for estimating the absolute (T) and relative (%T) penetration depth.
Figure 7.21 summarizes the absolute (T) and normalized (%T) depth of penetration measured for each nail and formulation.

Again, there was small inter-nail variability and no significant differences were found among the nails within each treatment group. Overall, NR penetrated to ~50 µm or 15% of the total thickness. The 20% PF-127 gel delivered NR deeper (T) into the nail plate than the 30% gel statistically, although the differences were quite moderate. As previously observed with SF, the discontinuous treatment resulted in NR signals that were noticeably less intense and with thinner fluorescence bands than those observed for continuous treatment samples.

### 7.3.3.3 Comparison between continuous and discontinuous application

As discussed above, the continuous, occluded application resulted in significantly deeper (T, ~1.8-fold) penetration of both fluorescent markers than the discontinuous, un-occluded approach (Figure 7.22). The difference was noticeable also in the confocal
dorsal images with the fluorescence signals being less intense and decreasing faster with depth for discontinuous dosing. This was not too surprising as the discontinuous procedure reduced to one-third the length of the nail exposure to the formulations compared to the continuous approach. In addition, the occlusive condition of the continuous approach results in enhanced nail hydration and probably facilitates diffusion of the markers (32). Yet, the continuous, occluded treatment did not triple the penetration depth and it could be hypothesized that some diffusion is still possible during the untreated (formulation-free) periods. In any case, the discontinuous approach reflects better the practical use envisaged for these formulation or daily overnight application. A word of caution is required as this work has used only healthy nail clippings so future research is required using diseased nails. Given the %T attained in 7 days, further optimization of these formulations will be required to promote drug permeation, maybe by incorporating a chemical enhancer (6, 33) or by wearing an occlusive layer on top of the formulation film to increase nail hydration (34).

![Continuous vs Discontinuous](image_url)

Figure 7.22: Penetration of SF and NR into the nail clipping following a 7-day continuous (C) and discontinuous (D) application with PF-127 (20%, 25% and 30%) gels. T (Mean + SD) values represent the average of 9 images obtained from 3 nails. The superscript letters indicate statistical difference (p < 0.05) between the pairs of bars (both T and %T).

### 7.3.3.4 Penetration pathways of SF and NR across the nail plate

An interesting finding of this work was the different penetration pathways followed by the two markers (Figure 7.23). As described in chapter 4, the red fluorescence of NR demarcated the onychocytes whilst the intracellular regions appeared darker. In contrary, SF was located predominately within the onychocytes. This difference could be related...
to the physicochemical properties of the two compounds, primarily their log P. It could be hypothesized that SF, being highly water-soluble (log P = -1.52 (35)), can access the more hydrophilic keratin-filled onychocytes. The involvement of the transcellular pathway was reported for SF and Nile blue chloride during passive and iontophoretic nail permeation experiments (29). NR, on the other hand, is highly lipophilic (log P = 4.07 (36)) and would follow predominantly the lipid-containing intercellular pathway.

![Figure 7.23: x-y planar images showing the localization of SF (left) and NR (right) at ~20 µm into the nail.](image)

The significance of the intracellular path contribution to nail passive permeation will require further corroboration. But, if confirmed, these findings would provide a distinct difference with regards to passive skin permeation during which transport of both hydrophilic and hydrophobic permeants shares the common continuous, intercellular pathway across the stratum corneum (SC) (37-39). This has been explained by the unique morphology of the SC, which is described as a construction of impermeable coenocyte ‘flakes’ incorporated into a lipid matrix and the formation of the so-called cornified envelope. The SC lipids lining the outside of the corneocytes are covalently bound by ester linkage to the outer surface of the cell membrane to provide a hydrophobic, impermeable layer. Diffusion across the SC intracellular pathway is slow and inefficient and the corneocytes do not provide an alternative diffusion path for hydrophilic molecules. On the contrary, by being inaccessible, the corneocytes increase the diffusional path and contribute to the barrier function (40). While the key role of the tortuous intercellular pathway has been well documented for the SC (41-43), little information exists concerning the nail plate. This work suggests the contribution of both the transcellular and intercellular paths to nail diffusion, and therefore that access to the
intracellular domains may be easier than in the SC. However, further investigation into
the structural and physical properties of the nail is required to provide a clear
mechanistic explanation. The findings here, nevertheless, provide useful information on
which future research can be based.

7.3.4 In vivo evaluation of PF-127

Table 7.5 summarizes the feedback from 18 volunteers concerning the use of PF-127-
based lacquer, and/or nail lacquers in general, as topical nail drug delivery systems.
Most participants responded favourably to the potential use of these formulations to
treat nail disease. Two participants found the formulations inconvenient to apply and the
drying time too long; further they perceived oral medications to be superior to topical
treatments. Surprisingly, the less frequent (weekly, every other day) dosing patterns
were not preferred and a daily application of the nail lacquer was considered acceptable
as far as “the formulation is easy to apply and forms the film rapidly upon application”.

Table 7.5: Summary of the feedback provided by volunteers on the use of nail lacquers
as a treatment option for nail diseases.

<table>
<thead>
<tr>
<th>Questions</th>
<th>Answers by participants (n out of 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consider nail lacquer as medical treatment</td>
<td>Yes (16), No (2)</td>
</tr>
<tr>
<td>Acceptability</td>
<td>No problem at all (10), Acceptable (6), Only if no alternative (2), Not at all (0)</td>
</tr>
<tr>
<td>Preferred dosing interval</td>
<td>Once daily (8), Every other day (5), Once weekly (5)</td>
</tr>
</tbody>
</table>

Placebo 20%, 25% and 30% PF-127 gels were applied to healthy human nail plates and
the gelling behaviour was examined. Table 7.6 summarizes the results. The three
formulations formed gels on the nail surface within 10 minutes post-application. The
physical appearance of the film was examined (Table 7.6). Bubbles were mostly
observed with films formed using 20% PF-127 and film coatings resulting from the
30% PF-127 were uneven, probably due to its high viscosity. Overall, the gel with 25%
PF-127 provided the best performance being highly rated for the shiny, homogenous
film appearance and absence of bubbles.
Table 7.6: Appearance of films formed following the application of placebo gels containing 20%, 25% and 30% of PF-127 to the nail plates of healthy volunteers.

<table>
<thead>
<tr>
<th>Film appearance</th>
<th>Participants (out of 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20% PF-127</td>
</tr>
<tr>
<td>Gel formed within 10 minutes</td>
<td>Yes</td>
</tr>
<tr>
<td>Good/Shiny</td>
<td>8</td>
</tr>
<tr>
<td>Contain bubbles</td>
<td>4</td>
</tr>
<tr>
<td>Viscous/Streaky</td>
<td>0</td>
</tr>
</tbody>
</table>

7.4 Conclusions

The potential use of 20%, 25% and 30% PF-127 thermoreversible gels as formulations for topical nail drug delivery was investigated using SF and NR as models for hydrophilic and lipophilic actives. The formulations provided a prolonged release *in vitro* profile for both markers with the release being faster for the 20% PF-127 gel. The formulations delivered both fluorophores into the nail plate as shown by LCSM imaging, following 7-day continuous and discontinuous applications. The latter mimicked a potential practical application of the formulations and, given the shorter exposure times, resulted in less penetration than the occluded, continuous treatments. Overall, none of the vehicles delivered the dyes across the whole nail thickness and the delivery performance of the three formulations was very similar. The confocal images suggested the transcellular and intercellular pathways to be predominant for SF and NR, respectively. Finally, the *in vivo* study suggested that PF-127 based thermogelling nail lacquers would be well accepted as topical nail treatments. The best *in vivo* thermogelling behaviour and film appearance was shown by the 25% PF-127 gel. Further optimization of these formulations is required to provide faster delivery of drugs and to investigate their performance on diseased nails.
Chapter 7

7.5 References


Chapter 8: Conclusions and Perspectives

In this thesis, the use of various non-destructive imaging techniques to assess nail permeation has been investigated. The objectives were to visualize and characterize the mechanism of drug uptake into the nail and, using this information, (a) to evaluate the potential of polymeric particle-based delivery systems for topical nail treatment; (b) to investigate the effect of microneedle and chemical enhancer treatment on drug uptake and permeation; and (c) to develop a new thermoreversible hydrogel-based formulation as a treatment option for topical nail diseases. The ultimate goal is to develop an underpinning science base with which to aid future improvement and optimization of topical nail formulations.

In this work, nails obtained from healthy human subjects were used. In vitro release and nail permeation experiments evaluated the performance of the different formulation approaches investigated. The thermoresponsive hydrogel was also tested in vivo to assess its practical usefulness. The nano- and sub-micron particles were prepared from various polymers and other constituents so as to achieve sustained release of the loaded 'active'; the particles were characterized for their size, morphology and entrapment efficiency. Laser scanning confocal microscopy (LSCM) and two-photon fluorescent (TPF) imaging enabled the localization of fluorescently-labelled nanoparticle reservoirs and the penetration of entrapped fluorophores (acting as model actives) on and within intact or microneedle-porated nails to be studied. Stimulated Raman scattering (SRS) microscopy, in contrast, tracked the diffusion of non-fluorescent compounds and permitted (semi-) quantitative analysis of their uptake kinetics into the nail. Delivery of an antifungal drug from sub-micron particles into and across the nail, and the effects of nail pre-treatment on drug uptake, were also evaluated. We now summarize the conclusions and perspectives of the various aspects of this research.

8.1 Stimulated Raman scattering (SRS) microscopy

SRS is a relatively new, label-free, high-resolution imaging technique based on the molecular vibrational frequency of a chemical bond of interest. In this thesis, SRS has been used for the first time to image the penetration of chemicals into the human nail in vitro. The time course of permeation of three solvents, water, propylene glycol and dimethyl sulphoxide, has been successfully tracked. Semi-quantitative analysis of the signal intensity indicated that each solvent altered its own diffusion into the nail in a
concentration-dependent manner, and thereby undermined the integrity of the tissue. Subsequently, SRS has enabled the release of a lipophilic compound, octyl methoxycinnamate, from polymeric nanoparticles (NPs) into the nail to be imaged. Although deuteration of the chemicals targeted for study (e.g., by replacing some or all C-H bonds by C-D) is not absolutely required, it is highly beneficial to improve image contrast because there is essentially no endogenous signal in the nail Raman spectrum at the relevant frequencies. An important future step is to validate a reliable correction procedure to account for the signal loss with imaging depth. This should ultimately allow the measured Raman signals to be directly correlated with the concentration of the chemical from which they originate. Together with further improvements in sensitivity, this will enable SRS to be broadly applicable to the study of the delivery of many different drugs from various formulations.

8.2 Laser scanning confocal microscopy (LSCM) and two photon fluorescence (TPF) imaging

LSCM and TPF are valuable techniques for the direct visualization of fluorescently-labelled samples. While fluorescence imaging has been extensively applied to various aspects of skin research, including percutaneous absorption, its use for investigations of the nail is much more limited. In this thesis, the LSCM and TPF techniques provided mechanistic insight into the distribution and transport pathways of fluorophores into the nail. The optical sectioning capabilities of the methods were complemented by examination of mechanically-sectioned nail samples. The accumulation and confinement of fluorescently-labelled nanoparticles on the nail surface and within microneedle-created pores was clearly observed. In contrast, the release and diffusion of the fluorescent marker, Nile red (NR), from the originally-loaded particles into the nail was demonstrable.

8.3 Pathways of nail permeation

The uptake of hydrophilic and hydrophobic fluorophores, sodium fluorescein (SF) and NR, respectively, into the nail was imaged using LSCM. The former penetrated mainly via transcellular pathways, whereas the latter diffused predominately via the lipid-containing intercellular regions, observations that have not been reported before. However, as only two compounds have been investigated, it is not yet possible to generalise about the preferred pathways of transport for compounds of specific
physicochemical properties. Nonetheless, the findings presented here form a useful basis for further fundamental research in this area.

### 8.4 Polymeric particle-based delivery

As anticipated, there was no evidence for particle penetration across the nail even the very small nanoparticles (only ~20 nm) were considered; there is simply insufficient free volume available, either trans- or inter-cellularly. The potential exists, however, for the particles to act as reservoirs for topical nail drug delivery and, in this research, the use of poly-methacrylate and poly(ε-caprolactone) particles was evaluated. For the former, the high affinity of the associated ‘active’ for the particles, and the high crystallinity of the polymer, prevented any test of the hypothesis. In contrast, sustained release of associated ‘actives’ (the lipophilic model compounds, NR and OMC) from poly(ε-caprolactone) particles and their distribution into the nail was demonstrated. The delivery of the antifungal drug, ciclopirox, into the nail from poly(ε-caprolactone) particles with an oil-filled core was also determined and the amounts taken up exceeded the minimum inhibitory concentration against the primary pathogens causing onychomycosis. Even though drug delivery into the nail from the particles was less than that from a marketed commercial lacquer formulation, the particles may offer sustained release of drug over a prolonged period of time whereas the later suffers from significant drug precipitation rapidly after application (due to the evaporation of volatile solvent). Future work on particle-based formulations should allow the applied ‘payload’ of drug to be increased and the methods used to enhance drug permeation across the barrier to be optimized.

### 8.5 Nail permeation enhancement strategies

Drug permeation across the highly keratinized nail structure remains a significant challenge. Physical and chemical enhancement methods, specifically microneedle-poration and penetration enhancer treatment, respectively, were evaluated. Further optimization of topical nail formulations may take advantage of these approaches.

Microneedle- poration created sequestration sites for nanoparticles from which the associated ‘active’ could be released and then diffuses into the deeper nail over a prolonged period of time. Additional work is required to control the consistency of pore size and depth.
N-acetyl cysteine, a sulfhydryl (SH)-containing amino acid derivative, cleaves disulphide bonds in the keratin matrix. Permeation of ciclopirox was increased significantly when nails were pre-treated with 10% N-acetyl cysteine prior to microneedle-poration.

Finally, hydration also promotes drug permeation by swelling the nail and increasing its porosity. A mechanism to maintain occlusion of the nail, therefore, should improve the performance of a formulation; alternatively, the concept of a ‘nail patch’ is perhaps worthy of consideration.

**8.6 Thermoreversible gel for nail delivery**

Finally, Pluronic PF-127 gels (at 20%, 25% and 30% w/v) as aqueous-based formulations for topical nail drug delivery were investigated. Prolonged *in vitro* release of (fluorescent) hydrophilic and lipophilic model actives from these gels was achieved and, as anticipated, the release was faster at the lower polymer concentrations used. LSCM imaging demonstrated the penetration of both fluorescent markers into the nail plate following topical application of the PF-127 formulations. However, the dyes did not permeate across the whole nail thickness during continuous or discontinuous treatment over 7 days and delivery from the three vehicles was very similar. *In vivo*, good acceptability and practicality of the PF-127-based thermogelling nail lacquers was demonstrated and further development is warranted, therefore, to increase the rate and extent of drug permeation from the gels (possibly taking advantage of the enhancement approaches examined here). Combination of the thermogelling lacquer with nano- or sub-micron particles to increase the substantiality of the formulation over prolonged periods may also be considered.
Appendices
Appendix 1: Ethical forms for nail collection
Participant information sheet

Collection of fingernails by nail clipping

You are being invited to take part in a research study. Before you decide on your participation, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please, contact us if there is anything that is not clear or if you would like more information. Take as much time as you want to decide whether or not you wish to take part in this study.

Thank you for reading this information sheet.

Purpose of the study:
The topical treatment of nail diseases, including fungal nail infections and nail psoriasis, is of limited success because of the poor permeability of the nail plate to drugs. The alternative is oral treatment (taken by mouth) but this exposes the whole body to the drug which results in frequent adverse effects and drug interactions.

Our research team is looking into new ways of applying drugs to the nail, including improving the nail formulations, using penetration enhancers and the application of iontophoresis (small electrical currents). We are also investigating the possible inclusion of these drugs into nanoparticles as one of these alternative new formulations. It has been shown that these nanoparticles act as reservoirs of drugs and increase the passage of drugs across the skin. Because skin and nails have some similarities we think that these nanoparticles might also have potential for improving the delivery drugs to the nails.

This study wants to answer the following questions:
1) Do any of these new formulations (nanoparticles, lacquers, patches) or enhancement methods (iontophoresis or chemical enhancers) improve the nail penetration of drugs commonly used to treat nail diseases (for example antifungal drugs such as ciclopirox olamine, amorolfine and terbinafine)?
2) Which properties of drugs determine how easily they permeated into the nail and which formulation or enhancement method works the best?
3) What types of formulation (solutions, gels, creams, and patches) are more appropriate to deliver the drugs to the nail?
4) How these new formulations compare to those available in the market? This will tell us whether they offer a practical benefit or not and if so, for which drugs.
This is the reason for performing this fingernail collection: To do these experiments we need samples of human nails. Hooves from animals, e.g. pig and cow are different in their structure and composition to human nails, so if we use them we may get the wrong answers to the questions above.

We require healthy volunteers to grow, and then donate their fingernail tips (8 mm length) to our research, so we can use the nails to perform experiments using different formulation techniques.

The participants must be healthy and their nails should not present sign of any disease, psoriasis, brittleness, infection, roughness (pitted nails) or decolouration. This is important because diseased and healthy nails may behave differently.

The chief investigator teaches to undergraduate pharmacy and pharmacology students. To avoid potential conflicts of interests, pharmacy and pharmacology undergraduate students at the University of Bath are also excluded from this study.

Your participation in the research:
It is up to you to decide whether or not to take part in this study. If you do decide to take part:
You will be given a copy of this information sheet to keep for your records, and there will be some time (from 1 day minimum to 2-3 months) during which you will let your fingernails grow. During all this time, you can change your mind about it; you are still free to withdraw at any time and without giving a reason.
Once the free edge of your fingernails has reached the minimal length required (8 mm), and if you still want to donate them; you will contact the research team.
Then, you will be asked to sign a consent form and a researcher will carefully cut the free edge of your nails. You may prefer to cut the nails yourself.

What is going to happen to you during the study?
If you agree to participate, the following will happen:
You will let your nails grow until their free edge has a length of at least 8 mm.
Please, note that you can choose how many (1 to 10) nails, in which hand, and which nails you prefer to let grow.
Depending on the starting length and individual differences, this growing period may take as much as 2-3 months. You can decide at any moment to stop your participation during this time and without giving a reason.
Once the free edge of the nails has reached the right length, and if you still want to donate them to our research, you will contact the research team. You will be asked to come to our laboratories (5W 3.22) at the University of Bath and you will be given a consent form to sign. After you have given your informed consent, a researcher (or yourself) will cut the free edge of your fingernails with a standard manicure scissors. Your nail clippings will be made anonymous and identified with a code: Subject 1/nail 1, Subject 1/nail 2 and so on. The researcher will fill a questionnaire to record the date of the donation, your name, age and gender, the finger and hand where each nail was harvested from and the use of nail cosmetics (if relevant). All this information will be kept separately from the nails so it will not be possible to identify the donor of each sample. The researcher will kept this information secure and only the research team will have access to it.
Once, the nail collection is done, you will be allowed to leave the laboratories and your participation will be considered as finished. The total donation act will take a maximum time of 30 minutes.
To acknowledge you for your time and contribution, we will pay you £4 per nail donated.
You may if you wish, participate more than once into the study.
**Lifestyle modifications:**
We would expect you to avoid the use of nail cosmetics (specially nail lacquers) while your nails are growing.
Some people may find unpractical or unpleasant to have long nails. You may decide to stop your participation at any moment, or just grow the nails you think more convenient.

**What will happen to my nails?**
Your nail clippings will be used for in vitro nail penetration studies.
Most of the experiments will study the penetration of antifungal or antipsoriatic drugs into the nails using different testing methods or formulations and compare the results with that obtained with the treatments available in the market.
Some other experiments will study the penetration of fluorescent compounds and/or deuterated compounds into the nail. We use these markers because they are easily seen under a microscope. Subsequently, the nails will be examined under a microscope to determine the depth of penetration of the marker.
By doing these experiments, we can tell if any of the proposed methods or the designed formulation is likely to result in an improved therapy of the nail diseases.
Your nail clippings will NOT be used for DNA analysis or any other test concerning your genetic inheritance.
At the end of the experiments the nail will be disposed of according to the regulations of the University of Bath that concerning the use of human tissues (autoclave and/or incineration).

**Can I get my nails back?**
If you agree to donate your nails to us, they will be considered as a gift and you will NOT retain any rights on them. For example, you can NOT claim them back. However, we will only use them for the research described in this form.

**Side effects:**
There are not side effects or risks expected from your participation into this study, other than those associated with nail clipping.
Some people may find unpractical or unpleasant to have long nails. You may feel inconvenient to have very short nails just after the nail clipping.

**Possible benefits of taking part:**
There is no direct benefit for you as a result of your participation. This is a preliminary study which will hopefully help us to develop more effective ways to treat nail diseases

**If something goes wrong:**
The University of Bath has site specific insurance to cover research on healthy volunteers. Please note that this insurance does NOT cover any harm that you could cause with your nails to yourself, others or any items during the growing period.

**Acknowledgement:**
To thank you for your participation and time we will pay you 4 pounds per nail collected at the end of the experiment.

**Confidentiality of taking part in this study:**
All personal information collected during the course of this research will be kept strictly confidential. When the results are made public, these will not include any names, initials, or any type of information which could result in your identification.
Appendix 1

Your nail clippings will be made anonymous and identified with a code: Subject 1/nail 1, Subject 1/nail 2 and so on. The researcher will record the date of the donation, your name, age and gender, the finger and hand where each nail was harvested from and the use of nail cosmetics (if relevant). All this information will be kept separately from the nails so it will not be possible to identify the donor of each sample. The researcher will kept this information secure.

Results of the study:
The results of this study will be published in thesis, scientific journals and/or presented during conferences, and/or internal reports. You will be allowed to have a copy of published articles upon request.

Organization and funding of the research:
This research is funded by the Department of Pharmacy and Pharmacology.
The protocol has been reviewed by the University Research Ethics Approval Committee for Health.

Contact for further information:
We will be more than happy to answer to all the questions you could have about this research and your participation.
Please contact:

Wing Sin CHIU: wsc21@bath.ac.uk  Phone: 01225 383900
Sarah Cordery: sc342@bath.ac.uk  Phone : 01225 384313
Dr Luis Nogueiras Nieto: l.nogueiras.nieto@bath.ac.uk  Phone : 01225383900
Dr Begoña Delgado-Charro: prsbd@bath.ac.uk  Phone: 01225 383969

You will be given a copy of this information sheet and a signed consent form to keep.

Thank you for considering taking part in this study.
CONSENT FORM

Title of study: Collection of finger nails by nail clipping.

Researcher: Miss W. S. Chiu / Miss Sarah Codery / Luis Nogueiras Nieto, PhD
Principal Investigator: Dr. M. Begoña Delgado-Charro

Please initial box

1. I confirm that I have read and that I understand the information sheet for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care, legal rights or relationship with the University being affected.

3. I understand that the donation of my fingernails is a gift. If I donate my nails to this study I will not retain any rights on them.

4. I agree to take part in the above study.

Name of Participant: ___________________________ Date: ______________ Signature: ___________________________

Researcher: ___________________________ Date: ______________ Signature: ___________________________

1 copy for participant, 1 copy for researcher
Questionnaire

Date:
1. Name: ..............................................
2. Subject code: .................................
3. Gender of the participant: ......................
4. Age of the participant: ........................
5. The donation concerns (circle nail code):
   
   L.F.1  L.F.2  L.F.3  L.F.4  L.F.5

6. Please, tell us if you used any nail cosmetics during the time you let your nails grow.
   ................................................................................................................................
   ................................................................................................................................
   ................................................................................................................................

7. Let us know if you had any previous nails infections or problems (brittleness, fungical infection, psoriasis, decolouration, etc).
   ................................................................................................................................
   ................................................................................................................................
   ................................................................................................................................

Thank you for your participation!
Appendix 2: Ethical forms for *in vivo* experiments
Participant information sheet

Can nail lacquers be formulated with thermo-gelling polymers?

You are being invited to take part in a research study. Before you decide on your participation, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please contact us if there is anything that is not clear or if you would like more information. Our contact details are at the end of this document. Take as much time as you want to decide whether or not you wish to take part in this study.

Thank you for reading this information sheet.

Purpose of the study:
The aim of our research is to examine whether a type of ingredients (thermo-gelling polymers) used in medicines preparation can be used to make nail lacquers. Nail diseases such as psoriasis and fungal infection are typically treated with oral medication, but this approach exposes the whole body to the drug which is not ideal. It would be much better to apply the medication only to the area affected that is, the nail, so the rest of the body is less exposed to the drugs and its possible adverse effects.

There are some medicated nail lacquers in the market which avoid this problem but their efficacy is limited and, for example, they can only be used in mild fungal infections. These nail lacquers are very similar to cosmetic nail varnishes and are based on organic solvents that evaporate upon application leaving a thin film on the nail plate. However, it is thought that when this happens the active drug forms crystals that, being trapped in the film, are not able to penetrate the nail. This chain of events renders the drug inactive in practical terms.

In our research group we are looking into alternative ways to formulate medicines to treat locally nail diseases. Ideally, the alternative formulation should remain on the nail plate after application and keep the drug in solution so it can be delivered to the nail.

Previous work suggests that aqueous formulations made with a special type of polymers (known as poloxamers or thermo-gelling polymers) could be an interesting choice. The aqueous solutions of these polymers have some interesting properties: they are liquid at low temperatures but can form a gel as the temperature increases. Potentially, they could be easily painted in the nails as a liquid, once in contact with the nail and as the temperature rises, they would form a thin gel film similar to a nail lacquer. Because these formulations are based on water soluble components they can be easily washed off. We envisage that a patient would apply them at night time and remove them in the morning simply by washing hands or showering. Studies on isolated nail clippings have shown that these formulations can deliver some drugs efficiently to the nail.
these studies cannot tell us whether they would form quickly a thin gel on the nail of people at normal body temperature. That is, whether they would be convenient to use in practical situations.

This study tries to clarify this point and will test the performance of un-medicated aqueous solutions of thermo-gelling polymers. To do this we plan to apply these solutions on the nails of healthy volunteers and observe their behaviour. We specifically want to answer these questions:

Are these formulations easy to apply? Do they spread easily on the nail?
Do these formulations form a thin gel in an acceptable time? If the time required is too long they would not be will not be practical.
How much of the solution is required for covering one nail?
What happens once these solutions are applied? Are the films aesthetically acceptable?

To do this we will recruit healthy volunteers and test the formulations on their nails. The formulations tested will not contain drugs as we primarily want to test their consistency and appearance once applied on the nail and this is mainly controlled by the thermo-gelling polymer.

Volunteer requirements:
To participate in this study you should, for your own safety and for the results of the experiment to be valid:
be healthy and not suffer from skin disease or allergies or nail disease.
not have any cosmetic treatment currently applied on your nails (for example, nail varnish, nail extensions, fake nails). You can participate if you have applied these treatments in the past.

Your participation in the research:
It is up to you to decide whether or not to take part in this study. If you decide to take part, please contact one of the researchers. Then:
1. You will be given a copy of this information sheet to keep for your records.
2. You will be asked to sign a consent form, you will be given a copy of the consent form.
3. If you change your mind, you are free to withdraw at any time and without giving a reason.

What is going to happen to you during the study:
- Your participation involves a maximum of ~30 minutes.
- The test, application of the formulation and observations, will take place in the research laboratories of the principal investigator (Dr Delgado-Charro) at the Department of Pharmacy & Pharmacology at the University of Bath (5W 3.22).
- You should not apply any nail cream or cosmetics (other than standard hygiene) to your nails before the study commences. You should not have any cosmetic treatment currently applied on your nails (for example, nail varnish, nail extensions, fake nails).

The step-by-step procedure will be:
1) You will be asked to fill a questionnaire (age, gender, recent use of nail cosmetic treatments or nail medicines, confirm your healthy status and lack of skin and nail allergies and diseases).
2) The researcher will apply the liquid formulation to all your nails. During this time we will ask you to seat and keep your hands steady, preferably flat on the desk.
3) The researcher will record the time required for the formulation to form a gel on the nail. If after 10 minutes this does not occur, or the gel formed is runny we will
abandon the experiment, you will receive you £5 acknowledgement, the formulation will be cleaned from your nails and your participation will stop. You are welcome to add your observations and comments to the questionnaire.

4) If the gel forms and remains steady on the nail, the researchers will examine its integrity and appearance and may take some pictures. These pictures will be restricted to your hands and nails and will not include your face.

5) You will be invited to comment on the use of medicated nail lacquers, on the films tested or the test in a questionnaire.

6) Your participation will be acknowledged with £5 and your participation will end.

7) Because the nature of the film (aqueous solution of water soluble components) it is expected that the film will be fast removed with just one routine hand washing. If you wish, you can remove the film from your nails before leaving the laboratories.

8) Should you, at any time, experience discomfort or feel unwell, the experiment will be stopped. You will still receive your £5 acknowledgment.

Possible adverse effects:
1) We do not expect that you will experience side effects because we will use aqueous solutions of components approved for preparing medicines and because the films will stay on your nails for a very short time. However, it is always possible for a participant to experiment some irritation or unexpected reaction or allergy to any chemical.

2) To limit any risk, the researchers will ensure that only the nail plate is painted with the formulation and will avoid the skin around the nail.

3) You must remain seated during the experiment while the formulation is applied, while we wait to see whether it forms a film on the nail and while we examine its behaviour and appearance. You may find this uncomfortable so we have limited the waiting time to maximum 10 minutes.

Possible benefits of taking part:
There is no direct benefit for you as a result of your participation. This is a basic research study designed to help us develop a new formulation to treat nail diseases.

Recognition of your time:
To thank you for your participation and time, we will pay you £5 at the end of the experiment. You will receive this amount even if the experiment is stopped before completion; for example, if you experience discomfort or the formulation fails to gel in 10 minutes.

Confidentiality
All personal information collected during the course of this research will be kept strictly confidential. When the results are made public, they will not include any names, initials or any type of information which could result in your identification. The researcher may ask for permission to take some photographs during the experiment. If you agree, the pictures will only include your treated hands and nails.

Results of the study:
The results of this study will be published in scientific journals and/or presented during conferences and/or internal reports. You will be allowed to have a copy of any published articles upon request.

Organization and funding of the research:
This research is funded by the Department of Pharmacy and Pharmacology-
Appendix 2

Undergraduate MPharm research projects fund.

The protocol has been reviewed and approved by the Research Ethics Committee for Health of the University of Bath.

Contact for further information:
We will be more than happy to answer any questions you have about this research and your participation. Please contact:
Principal investigator & supervisor:
   Dr. B. Delgado-Charro: prsbd@bath.ac.uk    Tel. 01225 383969
Postgraduate student:
   Wing Sin Chiu: W.Chiu@bath.ac.uk
Undergraduate students:
   Wing LEUNG: wtl28@bath.ac.uk    Wai Han KOH: whk25@bath.ac.uk
You will be given a copy of this information sheet and a signed consent form to keep.
CONSENT FORM for the study:

Can nail lacquers be formulated with thermo-gelling polymers?

Undergraduate Researchers: Wing LEUNG and Wai Han KOH:
PG student: Ms Wing Sin Chiu
Principal Investigator, PG and UG students’ supervisor: Dr. M. Begoña Delgado-Charro

1. I confirm that I have read and that I understand the information sheet dated 16/10/2013 (version 1) for the above study and have had the opportunity to ask questions

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected.

3. I understand that the researchers may take pictures of my hands and nails during the test. These pictures will NOT include my face.

5. I have not any skin or nail disease / allergy.

6. I have not any cosmetic treatment currently applied on my nails (for example, nail varnish, nail extensions, fake nails).

6. I understand that all the information I have provided will remain confidential and secure, under lock at Dr Delgado-Charro’s office after the study is completed and/or at the University of Bath computers/data storage systems. Anonymity will be kept so I will not be identified in any manner (through pictures, initials or names) in publication and reports resulting from this study.

7. I agree to take part in the above study.

Name of Participant          Date          Signature

Researcher taking consent form       Date          Signature
Study: “Can nail lacquers be formulated with thermo-gelling polymers?”

Questionnaire

Participant Identification Number:
Age: ……………. Gender: ……………

1. Do you have, or suspect you may suffer from any skin or nail allergy or disease? ……………………………………………………………………………
……………………………………………………………………………………

2. Are you currently wearing any cosmetic nail treatment (for example, nail varnish, nail extensions, fake nails)?

3. Would you consider nail lacquers (in general and the ones tested today):
   3.a. A user friendly way to apply medicines to nails? Y/N Why?
       ………………………………………………………………………………………
       ………………………………………………………………………………………
       ………………………………………………………………………………………

   3.b. Too much trouble/inconvenience? Y/N Why?
       ………………………………………………………………………………………
       ………………………………………………………………………………………
       ………………………………………………………………………………………

   3.c. Would you use them as a treatment if you had to apply them:
       Please choose one of these answers:
       Yes, no problem at all / acceptable / only if there is no alternative / Not at all.
       a. Once a day?
       b. Every other day?
       c. Once a week?

What do you think of the appearance of the films used?
……………………………………………………………………………………
……………………………………………………………………………………

Let us know if you have any other comments about the experiment:
……………………………………………………………………………………
……………………………………………………………………………………
Appendix 3: Confocal microscopy images
Figure 1: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 19LF1, (B) 1LF3 and (C) 16LF4. Scale bars = 50 µm.
Figure 2: Images of transversally sectioned nails: (A) 19LF1, (B) 1LF3 and (C) 16LF4. Scale bars = 200 µm.
Figure 3: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 19RF1a, (B) 16RF4 and (C) 2RF5. Scale bars = 50 µm.
Figure 4: Images of transversally sectioned nails: (A) 19RF1a, (B) 16RF4 and (C) 2RF5. Scale bars = 200 µm.
Figure 5: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 19RF1b, (B) 1LF2 and (C) 3RF5. Scale bars = 50 µm.
Figure 6: Images of transversally sectioned nails: (A) 19RF1b, (B) 1LF2 and (C) 3RF5. Scale bars = 200 μm.
Figure 7: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 10LF3, (B) 3LF1 and (C) 12LF4a. Scale bars = 50 µm.
Figure 8: Images of transversally sectioned nails: (A) 10LF3, (B) 3LF1 and (C) 12LF4a. Scale bars = 200 µm.
Figure 9: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 3LF5, (B) 1RF1a and (C) 12LF2. Scale bars = 50 µm.
Figure 10: Images of transversally sectioned nails: (A) 3LF5, (B) 1RF1a and (C) 12LF2. Scale bars = 200 µm.
Figure 11: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 5RF5, (B) 1RF1a and (C) 12LF4b. Scale bars = 50 µm.
Figure 12: Images of transversally sectioned nails: (A) 5RF5, (B) 1RF1a and (C) 12LF4b. Scale bars = 200 µm.
Figure 13: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 1RF2a, (B) 3RF2 and (C) 2RF5. Scale bars = 50 µm.
Figure 14: Images of transversally sectioned nails: (A) 1RF2a, (B) 3RF2 and (C) 2RF5. Scale bars = 200 µm.
Figure 15: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 10LF2, (B) 3LF2a and (C) 19RF5. Scale bars = 50 µm.
Figure 16: Images of transversally sectioned nails: (A) 10LF2, (B) 3LF2a and (C) 19RF5. Scale bars = 200 µm.
Figure 17: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 16LF3, (B) 3LF2b and (C) 19LF5. Scale bars = 50 µm.
Figure 18: Images of transversally sectioned nails: (A) 16LF3, (B) 3LF2b and (C) 19LF5. Scale bars = 200 μm.
Figure 19: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 19RF2, (B) 3LF3 and (C) 1RF2b. Scale bars = 50 μm.
Figure 20: Images of transversally sectioned nails: (A) 19RF2, (B) 3LF3 and (C) 1RF2b. Scale bars = 200 µm.
Figure 21: x-z orthogonal re-constructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 19RF3, (B) 3LF4 and (C) 12LF3. Scale bars = 50 µm.
Figure 22: Images of transversally sectioned nails: (A) 19RF3, (B) 3LF4 and (C) 12LF3. Scale bars = 200 µm.
Figure 23: x-z orthogonal reconstructions of dorsal images. Left panel: reflectance signal from the nail plate. Right panel: SF fluorescence. Images from nails (A) 12LF5, (B) 5LF4 and (C) 19RF4. Scale bars = 50 µm.
Figure 24: Images of transversally sectioned nails: (A) 12LF5, (B) 5LF4 and (C) 19RF4. Scale bars = 200 µm.