FORMULATION OF SOLID LIPID MELATONIN – LOADED MICROPARTICLES

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This thesis is presented for the Master of Philosophy of the University of Western Australia, School of Allied Health
2018
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1 Introduction

1.1 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine), a widely distributed molecule in nature, is a neurohormone secreted principally by the pineal gland. It is a naturally occurring compound that has been found in all life forms so far examined, from the simplest microorganism, to higher plants, and to the most complex life forms including man. Melatonin was isolated in 1958 from the bovine epiphysis by Lerner et al. (Lerner et al. 1958). Melatonin secretion follows the circadian pattern with high levels during the dark cycle. In addition to the pineal gland, melatonin is also synthesized in several other organs, for example in the enterochromaffin cells, the eye retina, peripheral teleneurons and the gastrointestinal tract. Its lipophilic nature enables it to enter the blood brain barrier (Johns 2011). Melatonin is not stored in any reserves, and thus the plasma concentration of melatonin reflects its rate of synthesis (Kasekar et al. 2014).

Pharmacological disruption of melatonin production can occur via beta-1 and alpha-1 receptors because of sympathetic innervation of the pineal gland. Its biosynthesis involves several steps. First, tryptophan is converted to serotonin (Webb & Puig-Domingo 1995). Then, acetylation by arylalkylamine N-acetyltransferase (AA-NAT), the rate limiting step in melatonin synthesis, is followed by conversion by the enzyme hydroxyl indole-O-transferase, into melatonin (Mulder et al. 2009). The suprachiasmatic nucleus of the anterior hypothalamus is responsible for the regulation of melatonin production (Srinivasan, Pandi-Perumal & Trahkt 2009).

Blood levels of the pineal hormone melatonin are high at night and low during the day. Its secretion is regulated by a rhythm-generating system located in the suprachiasmatic nucleus of the hypothalamus, which is in turn regulated by light. Melatonin is regulated not only by that circadian oscillator but acts as a darkness signal, providing feedback to the oscillator. Melatonin has both a soporific effect and an ability to entrain the sleep-wake rhythm. It also has a major role in regulating the body temperature rhythm (Brown 1994).

The circadian rhythm of pineal melatonin secretion is controlled by the suprachiasmatic nucleus (SCN) and is reflective of mechanisms involved in the control of the sleep/wake cycle. Melatonin is therefore very popular as a sleep remedy and a natural treatment for insomnia (Kasekar et al. 2014). It is proposed to promote sleep and the sleep/wake rhythm. Similarly, among individuals suffering from jet lag and shift workers, melatonin can assist in adjustments to work schedules and improve sleep quality (Pandi-Perumal et al. 2007). It might also be directly involved in the circadian timing of sleep (MacFarlane et al. 1991).
In both young and elderly individuals with primary insomnia, nocturnal plasma melatonin levels tend to be lower than those in healthy controls (Pandi-Perumal et al. 2007). For this reason, melatonin has been increasingly used to treat insomnia in children with attention-deficit hyperactivity disorders (ADHD) and autism as good sleep is essential for managing these conditions. There is also strong consensus among researchers that exogenous melatonin is beneficial for treating sleep-wake cycle disorders of children who have neurodevelopmental and neuropsychiatric difficulties (Jan et al. 2000; Gitto et al. 2011). In general, sleep onset latency is reduced significantly by melatonin while total sleep duration is increased (Brzezinski et al. 2005).

The lipophilic nature of melatonin allows its entry into cells and subcellular compartments, and to cross morphophysiological and blood brain barriers (Rodriguez et al. 2004). Although melatonin prolonged-release tablets are approved and being used for short-term treatment of primary insomnia characterised by poor quality of sleep in people aged ≥ 55 years (Wade & Downie 2008), it has also a potential to address other physiological processes, including reproduction, circadian homeostasis, retinal neuromodulation and vasomotor responses (Figure 1). In addition to regulation of the circadian rhythm, a variety of other physiological effects, such as hypnotic, anti-depressant, antiepileptic, oncostatic, immunomodulatory, anti-osteoporotic, anti-Alzheimer and neuromodulatory effects have been reported (Jamir Anwar et al. 2015; Haywood et al. 2009). Some of these effects will be discussed in more detail below.

**Figure 1. Pharmacological activities of melatonin (Hinkle 2002)**
1.1.1 Pharmacological actions of melatonin

1.1.1.1 Melatonin as an antioxidant

Tengattini et al. (2007) have reported evidence for the cardio protective effects of melatonin via its direct free radical scavenger and indirect antioxidant activity. Melatonin acts effectively against various reactive oxygen and nitrogen species and also modulates antioxidant and pro-oxidant enzymes. It has been reported to be an efficient scavenger of a variety of radical and non-radical reactants and can also improve the activity of glutathione peroxidase in the brain cortex as well as the gene expression for some antioxidant enzymes (Kotler et al. 1998).

1.1.1.2 Melatonin as an oncostatic

Melatonin is an extensive epigenetic modulator of gene expression and motivator of cell differentiation. Moreover, melatonin impedes aromatase activity by modulating the gene expression of specific aromatase promoter regions. It plays an important role in various stages of cancer development (tumour growth and metastasis) through different pathways and may thus have therapeutic indication in cancer treatment. Melatonin has been used, for example, as an adjunct to the routine chemotherapy of osteosarcoma to improve the prognosis of the disease (Jamir Anwar et al. 2015).

Melatonin has also been investigated as an agent against a wide range of other tumours (Jung & Ahmad 2006). The majority of these studies focus on hormone-dependant cancers related to disorders of the endocrine system including breast, prostate, uterus and cervical tumours. Impaired secretion of melatonin has been reported in patients suffering from breast, endometrial or colorectal cancer. In addition, the high incidences of breast and colorectal cancers observed in nurses and other night-shift workers suggest a potential correlation between cancer incidences and reduced melatonin secretion in these workers, in addition to their increased light exposure at night (Davis, Mirick & Stevens 2001).

The oncostatic action of melatonin has been linked to two main mechanisms. Melatonin may exert a protective effect by reversing cellular injury through neurohormonal regulation. It also has anti-cytoproliferative effects. Cellular metabolic processes and hence, adjustment of the antioxidant production in cells is regulated by cGMP and cAMP ratios. Melatonin deficiency leads to an uncontrolled cAMP synthesis, leading to unregulated oxidative processes and subsequent free radical damage (Fontenot & Levine 1990).
Several studies have shown melatonin to possess anti-gonadotropic and anti-oestrogenic actions against hormone-dependent tumours (Grant et al. 2009). Its antagonistic action against prolactin in human breast cancer cells has also been reported (Lemus-Wilson, Kelly & Blask 1995). Melatonin selectively neutralises the effects of estrogens on the breast and the local biosynthesis of estrogens from androgens. It also regulates the enzymes involved in the local synthesis of estrogens by modulating estrogen receptor expression (Mediavilla et al. 2010).

Interestingly, recent studies have suggested an increased expression of melatonin receptors in gastric adenocarcinoma tissues, and the marginal tissues in breast and colon cancers to indicate a refractory mechanism (Shokrzadeh et al. 2014).

1.1.1.3 Melatonin as a sleep inducer

Melatonin is a chronobiotic molecule that has been successfully used for the treatment of sleep problems related to perturbations of the circadian time keeping system, like those caused by jet lag and shift work. Melatonin has a positive impact on sleep disorders in shift workers as it modulates the circadian rhythm and improves their quality of sleep (Pandi-Perumal et al. 2008). It is also administered to counter jet lag due to changes in time zones during travel (Srinivasan et al. 2014).

Patients with metabolic syndrome exhibit sleep-wake disturbances and other circadian abnormalities, which may lead to rapid weight gain and development of diabetes and atherosclerotic diseases. Melatonin may have beneficial effects as it can change the phase and amplitude of circadian rhythms, and this has been shown to be beneficial in treating sleep disorders related to obesity (Laudon & Frydman-Marom 2014).

Melatonin is also efficacious in promoting better sleep in persons with primary insomnia by reducing sleep onset latency, increase total sleep time and improve overall sleep quality (Hansen et al. 2012). A meta-analysis of 17 different studies involving 284 subjects, the majority being elderly, concluded that melatonin was effective in increasing sleep efficiency and reducing sleep onset latency particularly in older individuals with nocturnal melatonin deficiency (Brzezinska et al. 2005). Not surprisingly, the British Association of Psychopharmacology (Wilson et al. 2010) recommends melatonin as the first choice treatment of primary insomnia in the geriatric population. It is, however, not a first line treatment for comorbid insomnia associated with a variety of neurological, psychiatric, cardiovascular and metabolic disorders.

In addition, sleep and the sleep-wakefulness cycle are influenced by melatonin via its action on receptors in the hypothalamic suprachiasmatic nuclei (SCN). In diurnal species, suppression of neuronal activity in the SCN is suggested to be the possible mechanism by which melatonin modifies
sleep. Melatonin has also been shown to influence sleep by changing the functions of the GABAA-benzodiazepine receptor complex; it improves both the amplitude and frequency of GABAergic inhibitory transmission (Cheng et al. 2012).

1.1.1.4 Melatonin as an antidepressant

Depression is often associated with impairment of sleep and circadian rhythms, and this has been linked to malfunctioned melatonin secretion (Quera Salva et al. 2011). As discussed, melatonin and melatonin agonists have chronobiotic effects, and are thus able to remodify the circadian system to treat seasonal affective disorders and mood disturbances (Sapede & Cau 2013).

Further, chronic melatonin administration at night-time was reported to significantly ameliorate stress-induced behavioural disturbances, especially cognitive dysfunction and depressive phenotypes (Haridas, Kumar & Manda 2013).

1.1.1.5 Melatonin as an antiepileptic

Treatment of epileptic disease is complex. Melatonin is a lipophilic molecule that can cross the blood brain barrier to modulate neuronal activity in the brain, and consequently, may have a useful role in the treatment of seizures. Melatonin reduces seizure latency and frequency, and improves electroencephalographic tracing (Forcelli et al. 2013). It appears to work by decreasing the glutamatergic and enhancing GABA neurotransmitters, and blocking voltage-sensitive Ca channels (Choi et al. 2014).

1.1.1.6 Melatonin as an immunomodulatory and anti-inflammatory agent

Melatonin is able, through its neuro-endocrine action, to reduce the inflammatory response to injured tissues (Malpaux, Migaud & Trioire 2001). Melatonin exhibited regulatory effects on both cellular and humoral immune responses. It does not only stimulate the production of natural killer cells, monocytes and leukocytes, but also alters the balance of T helper (Th-1 and Th-2) cells mainly towards Th-1 responses, and enhances the production of relevant cytokines, such as interleukin (IL)-2, IL-6, IL-12 and interferon-gamma.

Melatonin also plays an important role in epithelial functions. It is capable of protecting the gastrointestinal mucosa against damage by modulating the immune system, microcirculation and epithelial regeneration (Terry et al. 2009). Melatonin may also limit the mucosal damage in inflammatory bowel disease by inhibiting bacterial translocation and associated apoptotic effects, and reducing the level of tumour necrosis factor (Triantafillidis & Triantafillidis 2009).
1.1.1.7 Melatonin in cardiovascular disease

Melatonin lessens the molecular and cellular damage caused by cardiac ischemia or reperfusion in which destructive free radicals are involved. The anti-inflammatory and antioxidant properties of melatonin appear to be protective against hypertension, chronic vascular diseases, atherosclerosis and drug-induced cardiotoxicity (Jamir Anwar et al. 2015).

1.1.1.8 Melatonin in bone disease

Melatonin has bone anabolic as well as anti-resorptive effects by its direct action on osteoclasts and osteoblasts. A significant reduction in plasma melatonin concentration in humans after the age of 50 years has been associated with the progression of bone deterioration. The ability of melatonin to impede bone destruction and encourage bone healing in animal studies (Tresguerres et al. 2014) has led to suggestions that it may be used in bone grafting procedures, osteopenia, osteoporosis, and periodontal disease (Maria & Witt-Enderby 2014).

Melatonin supplementation to protect against osteoporosis was found to be well tolerated by premenopausal women who also reported improved physical symptoms (Jamir Anwar et al. 2015).

1.1.1.9 Melatonin as a neuromodulator

Activation of the melatonin receptors has been established to modulate the function of several types of neurons in the central nervous system by modifying the activity of ligand and voltage gated ion channels in rats (Zhao et al. 2010). Melatonin can enhance the activity of acetylcholine transferase in the frontal cortex and the hippocampus. It directly detoxifies free radicals and hence, increases the activity of acetyl choline in the rodent brain (Ahmed et al. 2013).

1.1.2 Endogenous Melatonin

![Biochemical pathway for the synthesis of melatonin](image)

*Figure 2. Biochemical pathway for the synthesis of melatonin (Hickman, Klein & Dyda 1999)*
Melatonin is an indole amide neurohormone primarily secreted by the pineal gland located behind the third ventricle in the brain. Melatonin is secreted from the pineal gland of all vertebrates during darkness and is the final product in the tryptophan metabolic pathway. As can be seen in Figure 2, serotonin is metabolized to N-acetyl serotonin by N-acetyl transferase (arylalkylamine N-acetyltransferase, or AANAT), and further methylated to melatonin by hydroxyl indole-o-methyltransferase (HIOMT) (Kasekar et al. 2014). HIOMT activity remains fairly constant, and the daily rhythm in melatonin synthesis is generated by a concurrent rhythm in AANAT activity (Hickman, Klein & Dyda 1999).

Melatonin production is not rate-limited by its immediate precursor molecule because the pineal gland contains high concentrations of serotonin. The pineal gland produces high levels of melatonin at night and this is regulated primarily through the β1-adrenergic receptors in the post-ganglionic sympathetic nerve endings, and to a much lesser extent by the parasympathetic nervous system that also activates the pineal gland. The primary stimulus for melatonin synthesis is norepinephrine (NE) release from the sympathetic nerve endings. In addition, stressful stimuli that trigger the release of NE from other sites can extend circulating catecholamine and stimulate daytime melatonin synthesis. Pharmacological disruption of melatonin production can occur via the β1- and α1-adrenergic receptors in the pineal gland. Melatonin biosynthesis is also inhibited by light and stimulated by periods of darkness independent of sleep.

Endogenous melatonin is rapidly eliminated from the circulation as a result of 6-hydroxylation in the liver, followed by excretion in a sulfatoxy-conjugated form. Melatonin t1/2 in plasma is about 10 min, thus, melatonin levels in the blood are directly reflective of the extent of melatonin production in the pineal gland (Jamir Anwar et al. 2015) (Figure 3).
Endogenous melatonin exerts its activity on daily and seasonal physiological rhythms by activating the melatonin receptors. To date, three G protein-coupled melatonin receptors have been cloned, as well as one nuclear receptor. Two of the G protein-coupled melatonin receptors have been widely investigated as potential therapeutic targets in disorders ranging from insomnia and circadian sleep disorders to depression, cardiovascular diseases and cancer (Dubocovich et al. 2010).

1.1.3 Exogenous melatonin

Commercial products are available that contain melatonin chemically synthesized from 5-methoxyindole or melatonin isolated from the pineal glands of cows. Melatonin is regulated as an active pharmaceutical ingredient in Australia. It is freely sold as a food supplement in USA, Europe and other countries across the globe. Melatonin was reclassified from prescription-only to Natural Health Product (NHP) status under the Canada Natural and Non-Prescription Health Products Directorate (NNHPD) in 2003. Melatonin has been available for over 20 years in the US dietary supplement market, where it is used by approximately 5% of the population. In 2011 the European Food Safety Authority (EFSA) considered the scientific opinion on the substantiation of a health claim related to melatonin and reduction of sleep onset latency (time taken to fall asleep). Melatonin was found to be sufficiently characterized and is now considered to be an API in Europe.

In general, animal and human data have indicated that the short-term use of melatonin is safe, even at extreme doses. Only mild adverse effects, such as dizziness, headache, nausea and sleepiness, have been reported. No studies to date have shown exogenous melatonin to induce any serious
adverse effects. Similarly, randomized clinical studies on long-term melatonin treatment have shown only mild adverse effects that were comparable to the placebo arm. The EFSA believes that the down scheduling of melatonin will have little or no adverse impact on public health. In one clinical trial, human volunteers instructed to consume 6 g of melatonin every night for 1 month reported minor stomach discomfort and residual sleepiness, but no major adverse events (Seabra et al. 2000).

1.1.4 Chemistry of melatonin

Chemically, melatonin is defined as N-acetyl-5-methoxytryptamine (Figure 4). It can be chemically synthesized via two different pathways using 5-methoxyindole as a starting material. Melatonin is a relatively low molecular weight hormone (M.W. 232.27 g/mol) and is a pale yellow crystalline material with a melting point of 116-120°C. It is soluble in water (0.1 mg/ml), ethanol (8 mg/ml), methanol, and dilute aqueous acid. Melatonin is stable in the presence of light, oxygen, water, 0.1 N HCl and 0.1 N NaOH solutions, its pKa is 4.4 and have a log P of 1.65 (Filalia et al. 2017).

![Figure 4. Chemical structure of melatonin (Kasekar et al. 2014)]

1.2 Rationale, Hypothesis and Aims

Melatonin is categorized by the US Food and Drug Administration (FDA) as a dietary supplement and is sold over-the-counter in the USA and Canada as immediate and prolonged release capsules and tablets mainly for improving sleep quality in elderly people. In 2007, the European Medicines Agency (EMA) granted marketing authorization for Circadin®, the first melatonin pharmaceutical product. In Australia, Circadin® is available as a TGA-approved prolonged release product for the treatment of
primary insomnia in patients older than 55 years. This exogenous melatonin has been used to manage sleep disturbances, including insomnia and jet lag (Petrie et al. 1989).

For children with ADHD, epilepsy, psychomotor retardation or other developmental delays, there is adequate evidence to show that an abnormal circadian melatonin rhythm with low blood level of melatonin at night is associated with sleep disturbances (Jan et al. 2000). Although melatonin is used off-label in paediatrics, it is not approved to be used in children in Australia.

Although melatonin administration has been shown to be effective in treating various human disorders, in particular improving the quality of sleep, the clinical use of melatonin is limited by a host of pharmacokinetic issues. $T_{\text{max}}$ is reached in approximately 50 min following the oral administration of melatonin, and its elimination from the body is rapid ($t_{1/2}$ of 1.509 ± 0.768 h). Moreover, the absolute bioavailability (BA) of melatonin is variable and low (Bartoli et al. 2013; Aldhous et al. 1985). Only 15% of the oral dose reaches the systemic circulation. The reasons for the poor BA have not been established with certainty, but have been postulated to be due to poor in vivo dissolution, low oral absorption, extensive first pass effect or a combination of these factors. Approximately 90% of a melatonin dose is cleared in a single passage through the liver. A small proportion of melatonin is also excreted unchanged in the urine (Harpsøe et al. 2015; Demuro et al. 2000; Bartoli et al. 2013).

There are several immediate and sustained release formulations of melatonin available in the market. However, the hypnotic effect of melatonin in immediate release formulations is not long enough to help patients stay asleep and hence, they usually suffer from nocturnal awakening (Albertini et al. 2014). Conversely, the sustained release products exhibit slow release of the active ingredient, and are unable to help patients who have difficulties in falling sleep (Albertini et al. 2014).

To solve these problems, the design of a new formulation to increase the solubility of melatonin in vivo and reduce its hepatic metabolism seems necessary in order to improve the bioavailability of a bolus release of melatonin. There is also a need to use a suitable carrier to sustain the release of melatonin in vivo in order to prolong the bioavailability. This would then produce a constant level of melatonin in the plasma over a prolonged period of time and therefore reduce the incidences of nocturnal awakening. Considering the rising prominence of solid lipid particles in developing novel pharmaceutical platforms, and the advantages of a spray congealing technique in their manufacturing, which will be discussed in the next section, microencapsulation of melatonin using
solid lipids prepared by a spray congealing method was considered to achieve the design of this formulation.

1.2.1 Encapsulation

Encapsulation relates to technologies which enable to formulate one active compound (or more), inside individualized particles with a specific geometry and properties. It is a drug delivery system based on microparticles with particle size ranges from one micron to few mm or nanoparticles with particle size ranges from one micron to few microns. This encapsulation technology allows protection of drug from the environment, stabilization of sensitive drug substances, elimination of incompatibilities, improving dissolution, controlling the release of active ingredient, or masking of unpleasant taste. Hence, it has played an important role in drug delivery systems aimed at improving the bioavailability of conventional drugs and minimizing side effects (Sharma et al. 2014).

In some cases, the microparticles are designed as microcapsules. A microcapsule is a system in which a drug containing core is completely surrounded by a polymer shell or lipid. The core can be solid, liquid or gas and the shell consists of a continuous, porous or non-porous layer (Pavan Kumar et al. 2011).

There are limited reported studies on the encapsulation of melatonin in microparticles. These reports used methods such as solvent evaporation or spray-drying (Schaffazickl et al. 2006). Albertini et al. (2014) have produced melatonin solid lipid microparticles by using a spray congealing method, and this formed the basis for this project.

1.2.2 Solid lipid microparticles as drug carriers

Solid lipid particles as products of microencapsulation, were introduced in the early 1990s as an alternative to traditional drug carrier systems such as emulsions, liposomes and polymeric microparticles for the encapsulation, targeting and controlled delivery of drugs and other actives. Microencapsulation is a coating technology applied to drugs to control their release into a specific medium or place at a known rate, reduce their toxicity and increase their solubility. The resultant microparticle is composed of the drug core or drug matrix, and the carrier material which is responsible for controlling the dissolution and release of the drug (Jackson & Lee 1991).
Solid lipid microparticles (SLM, Figure 5) are micro-scale drug carriers possessing a matrix made from fatty acid, glyceride, fatty alcohol or solid wax with high melting points (Gugu, Chime & Attama 2015). The lipids encompass a broad range of well-characterized pharmaceutical excipients that are biocompatible solid lipids at ambient temperatures, including triglycerides, partial glycerides, fatty acids, steroids and waxes (Mehnert et al. 2001). They combine many advantages of drug carrier systems. The amount of drug encapsulated can vary up to 95% for lipophilic and hydrophilic drugs and because they are made from physiological or physiologically related materials, they are well tolerated in living systems. The solid matrix protects loaded labile substances against degradation and it offers the possibility of controlled drug release and drug targeting (Umeyor et al. 2012). Compared to polymer microparticles, SLMs have the advantage of better biocompatibility, which minimizes the hazards of acute and chronic toxicity; they possess solid cores which reduce the mobility of incorporated drug and drug leakage from the carriers. They can be produced on a large industrial scale and are easy to produce (Milak, Medicott & G.Talker 2006). They also have the ability to mask the taste of some drugs and have been shown to enhance the absorption of both hydrophilic and lipophilic drugs and to protect the GI against the gastric irritation side effects of irritating medication (Chime et al. 2012).

**Figure 5. Structure of a typical solid lipid microparticle**

### 1.2.3 Hypothesis and aims

In recent years, there has been an active promotion of SLM as drug carriers. SLM have a large specific surface area, afford high drug loading and can be used to load lipophilic and hydrophilic drugs (Scalia, Young & Traini 2015). Compared to liquid lipid formulations, SLM significantly reduce the mobility of the drug load and are therefore better suited to provide a sustained drug release.
profile (Figure 6). Compared to polymeric systems, SLM feature better biocompatibility and significantly lower toxicity potential (Ekambaram, Abdul Hasan Sathlani & Priyanka 2012). Unlike the colloidal lipid systems, the physicochemical properties of SLM are less susceptible to change, giving these systems good stability and longer shelf lives (Umeyor et al. 2012). Moreover, the production of SLM does not involve the use of noxious organic solvents, can be readily scaled up and is generally of low cost (Mader, Gohla & Muller 2000).

In particular, the effect of lipid microparticle carrier systems on the light-induced degradation of melatonin can be an advantage. Photolysis experiments have shown that the light-induced decomposition of melatonin was greatly reduced by encapsulation into lipid microparticles. Therefore, lipid encapsulation can be considered an effective system to enhance the photo stability of melatonin (Tursilli et al. 2006).

The hypothesis for this project was that melatonin-loaded SLM could be successfully fabricated using the Buchi Encapsulator, and that a pulsatile system characterized by a surge cum sustained release

Figure 6. Advantages of solid lipid microparticles over emulsions and liposomes (Umeyor et al. 2012)
of melatonin could be produced using a combination of immediate-release and sustained-release melatonin-loaded SLM. The rationale for adopting a SLM approach is that these particles have very good prospects for effective encapsulation of melatonin and controlling its rate of release.

The specific aims of this project are:

(1) To optimise the production of SLM via the spray congealing method using the Buchi™ Encapsulator.
(2) To optimise the fabrication process for melatonin-loaded SLM for different solid lipids.
(3) To characterize the properties and drug release kinetics of the optimised melatonin-loaded SLM.
(4) To determine whether a surge-sustained pulsatile system can be produced using combinations of melatonin-loaded SLM.

1.3 Preparation of solid lipid microparticles

There are different techniques for the preparation of SLM. Generally, the methods require a dispersed system as precursor or template, otherwise the SLM are produced through the use of a particular equipment. The most important methods for the preparation of SLM are described briefly in the following sections. At the end, the spray congealing method and the reasons why we chose this particular method in this study are also described.

1.3.1 Emulsion precursors

An emulsion consists of a mixture of two immiscible liquids, one of which is dispersed as droplets in the other with the help of an emulsifying agent. In techniques using emulsion precursors, the solid lipid is heated to 5-10 °C above its melting point to obtain a liquid which is emulsified with equally hot water under agitation. The emulsion is then cooled to room temperature with continuous stirring to allow the suspended lipid droplets to solidify into microparticles. SLM can be prepared by this melt dispersion method using simple O/W (oil-in-water) emulsions or complex W/O/W emulsions (Battaglia et al. 2014).

1.3.2 High pressure homogenization (HPH)

In HPH, the solid lipid is heated to above its melting temperature, and the molten lipid is forced through a narrow nozzle under high pressure in a homogenizer. The fluid speeds up over a very short distance to very high velocity. Very high shear stress and cavitation forces disrupt the fluid flow,
causing the breakup of particles down to the micron size range. Ultrasonication may also be applied to yield smaller particles. Two general approaches are available, hot homogenization and cold homogenization, both of which involve mixing the drug in the bulk of the lipid melt (Yadav et al. 2014).

Hot homogenization (Figure 7) is performed at temperatures above the melting point of the lipid. Higher temperatures result in lower particle sizes due to the decreased viscosity of the liquid phase. However, high temperatures increase the potential for degradation of the drug and the carrier. Increasing the homogenization pressure or decreasing voltage often results in an increase of the particle size for this method (Umeyor et al. 2012).

The HPH method is versatile and applicable to many drugs and lipids. It does not use noxious organic solvents, which is advantageous from a toxicological perspective. However, HPH exposes the drugs and lipids to high temperatures and pressures, which can induce thermodynamic and mechanical stresses that are detrimental to thermolabile drugs. Furthermore, drug expulsion can occur as the lipid recrystallizes on cooling, and this can lead to SLM with low drug loading (Battaglia et al. 2014).
Cold homogenization (Figure 8) was developed to replace hot homogenization as a SLM preparation method for thermolabile drugs. In this method, the drug-loaded molten lipid is size reduced to the micron size range, emulsified in water, then allowed to cool to form a suspension of particles which is then forced through the high pressure homogenizer to obtain smaller particles of the desired size range (Mehnert et al. 2001).
1.3.3 Microemulsion method

Microemulsions are two-phase systems containing an inner phase and an outer phase and are prepared by stirring mixtures of a low melting fatty acid, emulsifier, co-emulsifiers and water at 65-70°C. The hot emulsion is dispersed with agitation into cold water to facilitate rapid lipid crystallization and prevent aggregation (Figure 10). Due to the dilution with water, the yields are significantly lower in comparison with the HPH process (Mukherjee, Ray & Thakur 2001). Compared to the emulsion methods, the size and shape of the particles produced from microemulsions are much smaller, often in the nanosize range.
Microemulsions are thermodynamically stable systems and, unlike emulsions, they do not require high energy input for preparation. SLM fabricated via microemulsions have attracted increasing attention as drug delivery systems due to their capacity to achieve high drug loading for poorly water-soluble drugs (Battaglia et al. 2014).

1.3.4 Solvent evaporation methods

SLM can also be produced by solvent evaporation methods. In this case the lipid is dissolved in a water-immiscible organic solvent and emulsified in an aqueous phase by high pressure homogenization. Upon evaporation of the organic solvent under reduced pressures of 40-60 mbar, the lipid droplets precipitate to give a SLM dispersion in the aqueous layer (Mehnert et al. 2001).

SLM can also be prepared using an organic solvent that is volatile or partially water miscible. The emulsion precursors may be O/W or W/O/W systems. O/W emulsions are used for lipophilic drugs that are dissolved in the inner organic phase of the system, together with the lipid. W/O/W emulsions are suitable for hydrophilic drugs that are dissolved in the inner aqueous phase, while the lipid is dissolved in the intermediate organic phase of the multiple system. Particles are formed when the organic solvent is removed either by evaporation or by water dilution (Battaglia et al. 2014). Of the two, the solvent evaporation technique has many drawbacks due to the toxicity of the chlorinated solvents that are commonly employed. Solvent diffusion induced by the addition of
more water is a more acceptable method, and most of the solvents employed in this technique show a better safety profile compared to the volatile solvents.

Figure 10. Solid lipid microparticle production by emulsification-diffusion (Ekambaram, Abdul Hasan Sathlani & Priyanka 2012)

1.3.5 Supercritical fluid method

Supercritical fluid (SCF) technology has attracted increasing attention in recent years for SLM production. A SCF of a material is obtained above its critical pressure and temperature, and is exploited for its high capacity to dissolve other materials. Due to its low critical point at 31 °C and 74 bar, and its low cost, carbon dioxide (CO₂) is the most widely used SCF to fabricate SLM (Berton, Piel & Evrard 2011).

The four main SCF-related techniques are:

1. Rapid Expansion of Supercritical Solutions (RESS)
2. Gas Anti-Solvent (GAS) process
3. Gas Assisted Melting Atomisation (GAMA)
4. Supercritical Fluid Extraction of Emulsions (SFEE)
In RESS the materials are dissolved in the SCF, which is then expanded through a nozzle to produce the particles. RESS is of limited application to SLM fabrication because the relative low solubility of compounds in the SCF precludes production at reasonable costs.

The GAS process has been developed for hydrophobic materials that cannot be processed by the RESS technique due to their poor solubility in the SCF. In GAS the SCF solution is expanded sufficiently by introduction of a gas, which causes nucleation for precipitation of the solutes (Byrappa, Ohara & Adschiri 2008). The GAS technique is also of limited application for SLM production.

The GAMA technique involves melting the lipids in a thermostated mixing chamber (CM), mixed with SCF and forcing them through a nozzle at the bottom of the CM to produce SLM. The particles are gathered and dispersed in water by vortexing and sonicating, with polyethylene glycol (PEG) added to aid their dispersion in the water phase (Battaglia et al. 2014).

In SFE, fine O/W emulsions are prepared by dispersing a solution of lipid and drug, dissolved in a volatile organic solvent, into an aqueous solution of surfactant using a high pressure homogeniser. The emulsions are introduced into the top of an extraction column while the SCF is introduced in a counter direction from the bottom. SCF extraction of the organic solvent transforms the O/W emulsions into SLM suspensions (Chattopadhay et al. 2007).

1.3.6 Spray drying method

SLM fabrication by spray drying (Figure 11) is recommended for lipids with melting points of more than 70 °C (Ekambaram, Abdul Hasan Sathlani & Priyanka 2012). Spray-drying is a one-step process in which a liquid feed (solution, suspension or colloidal dispersion) is atomised into a stream of hot gas, resulting in the rapid evaporation of the solvent to form dried solid particles (Manu et al. 2012).
The major benefit of the spray-drying technique is the capacity to design SLM of specific size distribution, morphology and density by manipulating operational parameters, including solvent composition, solute concentration, solution and gas flow rates, temperature and relative humidity. However, the spray-drying process may induce degradation of some macromolecular drugs as a result of thermal stress during droplet drying and high shear stress in the nozzle (Pilcer & Amighi 2010).

1.3.7 Spray congealing method

In the spray congealing technique (Figure 12), lipids are heated to a temperature above their melting point and the drug is dissolved or suspended in the molten lipid. The hot mixture is then atomised through a pneumatic nozzle into a vessel, where the atomised droplets can solidify in the form of microparticles.

There are generally two different nozzles. The Wide Pneumatic Nozzle (WPN) is an external mixing atomiser, and atomisation occurs when the air input converges with the molten fluid outside of the nozzle. The Air Pressure Nozzle (APN) is an internal mixing device. The swirling molten fluid interacts with the atomising air to create extreme turbulence in the chamber before flowing through an orifice under shear, and leaving the nozzle as a finely atomised spray cone (Passerini et al. 2010).
Another atomiser is based on the application of ultrasound (Battaglia et al. 2014) to create a vibrating surface (sonotrode) on which the atomisation of the liquid occurs. The atomiser also contains an inductive coil to keep the sonotrode at suitable temperature. The molten lipid mixture, fed to the sonotrode by a thermostated reservoir through a funnel, is atomised by ultrasound energy into small droplets that fall freely and solidify by cooling at room temperature.

In the last two decades, spray congealing, also called spray chilling or spray cooling, has attracted increasing attention because this technique does not require the use of organic or aqueous solvents and hence is environmentally friendly. It also requires less time and energy consumption compared to other methods. Spray congealing is a very versatile technique due to the fact that both hydrophilic and hydrophobic low melting point carriers can be used (Passerini et al. 2010). The selection of a suitable carrier permits to mask the bitter taste of drugs or to modify the dissolution behaviour of the active pharmaceutical ingredients for oral administration: hydrophilic carriers have been utilized to enhance the dissolution rate of poorly water soluble drugs while hydrophobic carriers have been employed to control the release of short half-life drugs (Passerini et al. 2010).
2 Method development to produce melatonin-loaded solid lipid microparticles using the Buchi Encapsulator

2.1 Introduction

This project utilized the Buchi™ Encapsulator B-390 to manufacture the melatonin-loaded SLM by the spray congealing technique. The encapsulator (Figure 13) works on the principle that a laminar liquid jet can be broken up into similarly sized spherical droplets by applying mechanical vibration. A controlled, superimposed vibrational frequency is applied to break the liquid feed jet into small, uniform droplets of equal size, with one droplet formed per Hertz of frequency applied. The Buchi™ Encapsulator has been validated to produce round polymeric microparticles by the coacervation technique, e.g. by atomising a solution of sodium alginate into droplets that are guided into a calcium chloride bath for subsequent solidification into microparticles. There is little experience in using the encapsulator for fabricating SLM, so this was a core aim of this part of the project.

Encapsulation can be defined as a process which involves the complete envelopment of preselected materials within a matrix or membrane to give miniature sized particles preferably spherical in shape. In this part of the study, we aimed to adapt the Buchi™ Encapsulator to produce immediate release SLM loaded with 2.5% w/w of melatonin. The loading of 2.5% w/w of melatonin was chosen as it would enable the incorporation of the SLM into tablets of reasonable weight and containing a range of melatonin strengths from 0.5 mg to 10 mg. Three waxes, Gelucire 50/13, Witepsol H15 and hard paraffin were used as the starting lipid materials as they are common pharmaceutical materials generally regarded as safe. The oral LD$_{50}$ of paraffin is > 5000 mg/kg in rat (Safety Data Sheet 2015) and Witepsol H15 has an oral LD$_{50}$ of >2000 mg/kg in rat (Material Safety Data Sheet  2016). More importantly, they present an appropriate range of melting temperatures (30 - 70°C) that allow to evaluate the feasibility of fabricating melatonin-loaded SLM using the Buchi™ Encapsulator.

Gelucire 50/13 is a semi-solid lipid comprising glycerides and a mixture of PEG1500 (parent compound and mono- and di-esters). It is a non-ionic, water-dispersible surfactant with a melting point of 37 – 60 °C. Gelucire 50/13 is employed as a solubilizer and wetting agent, as well as an emulsifier for self-emulsifying drug formulations. It has good thermoplasticity for use as a binder in melt processes. Gelucire 50/13 has been chosen for this project because SLMs prepared with this lipid have the potential to improve the dissolution and bioavailability of poorly water-soluble drugs like melatonin (Sheng, Marchaud & Craig 2010).
Witepsol H15 consists of solid triglycerides manufactured through the direct esterification of glycerol with defined fatty acid blends, and has therefore more precise properties regarding its melting point, polarity and consistency compared to naturally derived lipids. Witepsol H15 H grades are hard fats with a low hydroxyl value (≤ 15), and a maximum of 15% triglycerides and 1% monoglycerides. They are white to off-white odourless pellets, greasy to the touch, practically insoluble in water and slightly soluble in anhydrous ethanol. The melting temperature of Witepsol H15 is 31 – 38 °C (Material Safety Data Sheet 2016).

Hard paraffin wax is a white or colourless soft solid derived from petroleum, coal or oil shale consisting of mixtures of hydrocarbon molecules containing 20 – 40 carbon atoms. It is insoluble in water. Hard paraffin typically has a melting point between 46 - 68 °C (Rowe, Sheskey & Quinn 2014).

![Figure 13](image-url)

*Figure 13. (a) The Buchi™ B-390 (b) Nozzle and nozzle orifice*
2.2 Materials and Methods

2.2.1 Materials

Melatonin was provided by Medisca Inc. (Plattsburgh, NY, USA); Gelucire 50/13 USP pellets were donated by Gattefosse SAS (Saint-Priest, France); hard paraffin white was purchased from Sigma-Aldrich Laborchemikalien GmbH (Darmstadt, Germany); Witepsol H15® was supplied by PCCA (Houston, Texas), and glycerine was obtained from Chem-supply Pty Ltd (Gillman SA, Australia). All other materials were of analytical grade. Solvents for HPLC were of HPLC grade. Deionised water (BOSS 031-4P) was used throughout. Phosphate buffer of pH 7.4 was prepared as per the European Pharmacopeia 7th edition (European Pharmacopeia 2011). HCl solution of pH 1.2 was prepared as per the European Pharmacopeia 7th edition as well (European Pharmacopeia 2011).

2.2.2 Preparation of solid lipid microparticles

Accurately weighed Gelucire 50/13 (97.5 g), Witepsol H15 (97.5 g) and hard paraffin (97.5 g) were separately heated to 10°C above their melting points in separate beakers on a hot plate stirrer from IEC Industrial Equipment and Control PTY LTD, CS 76083V (Melbourne, Australia). While stirring, 2.5 g of melatonin was added to each of the molten waxes to yield uniform mixtures (Albertini et al. 2014). Each mixture was then added into the feeding chamber of the encapsulator to be extruded into liquid droplets and solidified into SLM. The time taken to extrude 100 g of the melatonin-lipid mixture was about 2-3 min.

Preliminary experiments had established that the size of fabricated SLM depended on several processing parameters, including the pressure of the nitrogen gas, the degree of mechanical vibration, the size of the nozzle and the temperature of molten lipids. To fabricate the SLM, the connecting tubes and nozzle of the encapsulator had to be maintained at 10°C above the melting point of the respective lipid by controlling the temperature on the encapsulator and hot plate. To avoid the solidification of the molten lipid in the tubes and nozzle orifice the molten drug-lipid mixture in the feed bottle was also maintained at 10°C above the specific lipid melting point using the hot plate stirrer to minimize premature lipid solidification, drug segregation, and the production of SLM with non-uniform melatonin content. A nozzle with orifice diameter of 1 mm was used to fabricate the SLM.

The molten mixture was pumped into the nozzle by applying a nitrogen gas pressure of 2 bar. Optimal atomization of the molten liquid as it emerged from the nozzle was aided by adjusting the
vibrational frequency to 700 Hz and applying an electrostatic charge. The desired outcome was the production of a cone of small, round and evenly sized liquid droplets. The droplets were partially solidified as they descended, and fully solidified when they entered into a coolant bath placed beneath the nozzle.

Two litres of ethanol, iced water, glycerol and vegetable oil, respectively were used as coolant baths and stirred at 50 rpm in a five litre beaker using a magnetic stirrer. To determine the optimal cooling conditions for the molten lipid droplets, the distance between the nozzle and the coolant bath was maintained at 40 cm, 70 cm and 200 cm (Figure 14). After the last molten lipid droplet was extruded, the particles were allowed to stir in the coolant for 5 min. The fabricated SLM were then recovered from the coolant by filtration (11 µm, Whatman filter paper) and dried overnight at ambient temperature. They were then stored in glass bottles at ambient condition, and analysed within a month of manufacture.
Figure 14. Placement of encapsulator and coolant bath to achieve the separation distance of (a) 40 cm; (b) 70 cm; and (c) 200 cm.
2.2.3 Characterization of the solid lipid microparticles

2.2.3.1 Particle size analysis

The size distribution of microparticles was evaluated using an OLYMPUS CKX41 optical microscope, Olympus Australia Pty Ltd (Notting Hill Victoria 3168, Australia) with magnification at 10×0.25. Microparticles were randomly sampled from each batch of SLM, suspended in water on a microscope slide, and covered with a coverslip. The diameter of 100 microparticles were measured for each batch with the aid of a stage micrometre.

2.2.3.2 Morphological Analysis

The morphology of the SLM was analysed under a scanning electron microscopy (SEM), Zeiss 1555 VP-FESEM (VIC, Australia). The samples were coated with carbon 30 nm and examined at a voltage of 15 KV, span speed of 9, aperture size of 30 µm and magnification of 68x.

2.2.3.3 Assay of melatonin content using High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of two mobile phase delivery pumps, a UV-Vis detector and an auto sampler from Agilent Technologies 1260 Infinity (Santa Clara, California, USA). Samples were injected into a C18 column (5 µm, 150×4.6 mm, Thermo Scientific ODSHUPersil). The HPLC was calibrated with melatonin standard solutions prepared by dissolving 4 mg of melatonin in 100 ml of the mobile phase and diluting this stock solution with the mobile phase to give working solutions with melatonin concentrations of 1, 5, 10, 15, 20 and 40 µg/ml. The mobile phase consisted of ammonium dihydrogen phosphate buffer (0.02M, pH 2.85) and methanol in the volume ratio of 65:35 v/v. The assay was conducted using an isocratic mode with a flow rate of 1 ml/min, a detection wavelength of 230 nm and a run time of 20 min.

The HPLC was validated for the assay of melatonin by determining the following parameters: precision, limit of detection (LOD) and limit of quantification (LOQ). To determine precision and accuracy, melatonin standard solutions were prepared at concentrations of 1, 20, 60 µg/ml and each was assayed 3 times on a single day to determine the intraday coefficient of variation, and once daily on 3 consecutive days to determine the interday coefficient of variation (Lawal et al. 2012). LOQ is the lowest amount of melatonin in a sample which can be quantitatively determined with adequate precision and accuracy. LOD is the lowest amount of melatonin in the sample which can be detected but not necessarily quantified. LOQ and LOD were calculated according to Equations 1 and 2, respectively (Lawal et al. 2012).
LOQ = 10 (SD/S) \hspace{1cm} \text{Equation 1}

LOD = 3.3 (SD/S) \hspace{1cm} \text{Equation 2}

Where SD is the standard deviation of the response and is determined as the y-intercept of the linear calibration curve, and S is the slope of the calibration curve. A total of 5 calibration curves were plotted for LOQ and LOD determinations.

To determine whether the HPLC assay was stability-indicating, 4 ml of the high concentration melatonin standard solution (60 µg/ml in phosphate buffer) was mixed with 1 ml of 0.1 M HCl and boiled for 30 s and 2 min. The solutions were then analysed using the above HPLC assay to determine whether the degraded products elute at the same time as the parent drug.

The analysis of melatonin content in the microparticles was determined using a method modified from that described by Albertini et al. (Albertini et al. 2014). A 120 mg aliquot of microparticles was sampled from each batch of melatonin-loaded SLM and placed in 200 ml of phosphate buffer solution (PBS) pH 7.4. The suspension was heated with agitation to 10°C above the melting point of the lipid carrier, and the resultant solution was filtered (0.2 µm, Phenomenex, Thermo Scientific), diluted ten times with the mobile phase, and injected at 20 µl into the HPLC for analysis (Albertini et al. 2014). Each batch was analysed in triplicates and the mean ± SD of melatonin content was calculated. Melatonin loading in the SLM was expressed as the mass of melatonin per unit mass of SLM (mg/g).

The encapsulation efficiency (EE %) was calculated according to Equation 3 (Emma Piacentini 2016).

\[
\text{EE\%} = \left( \frac{\text{Wt}}{\text{Wi}} \right) \times 100
\]

\hspace{1cm} \text{Equation 3}

where Wt is the amount of melatonin determined for each 100 mg of sampled SLM (mg/100 mg of SLM) and Wi is the amount of melatonin used for the fabrication of 100 mg of SLM (here 2.5 mg/100 mg of SLM).

As it was suspected that in the fabrication of SLM, melatonin might have diffused from the hardening SLM into the ethanol coolant bath, after the hardened SLM were decanted from the coolant bath, the melatonin content in the bath was determined by UV-VIS spectrophotometry. The Varian 50-Bio UV-Vis Spectrophotometer (Agilent Technologies, Mulgrave, VIC, Australia) was calibrated using standard melatonin solutions (0.5 to 4 µg/ml in ethanol) at 230 nm.
2.2.4 Melatonin stability

Melatonin was supplied with labelling instructions to store under refrigeration. As the fabrication and characterization of the SLM involved the exposure of melatonin to heat, acid and alkali, it was necessary to determine whether melatonin was stable to these conditions. To determine its heat stability the drug was assayed after it was subjected to heating at 90°C for 2h. The drug was presented in the following forms for the heat treatment: 5 g melatonin powder; 5 ml of melatonin standard solution (60 µg/ml of melatonin in phosphate buffer); 4 ml of melatonin standard solution mixed with 1 ml of 1 N HCl; and 4 ml of melatonin standard solution mixed with 1 ml of 1 N NaOH. The powder was presented on a glass petri dish and the solutions in 20 ml amber glass vials. All heat treated samples were assayed immediately after exposure for their melatonin content using the established HPLC method.

2.3 Results and Discussion

2.3.1 Encapsulation method development

The Buchi Encapsulator was applied to fabricate melatonin-loaded SLM using three different types of solid lipids, namely Gelucire 50/13, Witepsol H15 and hard paraffin. Each batch of SLM was produced using 97.5 g of lipid and 2.5 g of melatonin, and a single axial nozzle with an orifice diameter of 1 mm. The jet of molten lipid emerging from the nozzle was transformed into a cone of uniformly sized liquid droplets with the help of vibration forces.

First the use of a coolant bath to solidify the molten hard paraffin droplets emerging from the nozzle was investigated. Different coolant bath with different viscosity and surface tension were examined to find out which of those produced the best spherical particles. Iced water was initially used as coolant. The distance between the nozzle orifice and the surface of the water bath was set at 40 cm. The molten hard paraffin droplets were observed to spread out on contact with the water, and to congeal into an amorphous mass on the water surface (Figure 15). When the iced water was replaced with glycerol and vegetable oil as coolant, the same result was obtained. However, when ethanol at ambient temperature was employed as the coolant, a considerable amount of the lipid droplets were successfully solidified into SLM, although small areas of amorphous hard paraffin were still visible on the surface of the ethanol bath.

The effectiveness of the 4 coolants for the fabrication of hard paraffin SLM might be related to their relative surface tension and density. The surface tension of the coolants decreased in the order of
water (73 dynes/cm at 20°C) > glycerol (64) > vegetable oil (34) > ethanol (22) Surface tension is the net inward force acting along a liquid surface. It causes the liquid to acquire the least surface area possible and to behave as if its surface is a stretched elastic membrane. The higher the surface tension, the stronger the surface elasticity, and the more effective the coolant was at supporting the hard paraffin liquid droplet on its surface. Hard paraffin liquid droplets that remained on the coolant surface adhered, spread out and congealed into an amorphous mass. Similarly, when considering liquid density, it can be anticipated that the hard paraffin liquid droplets would float on the surface of an immiscible liquid of higher relative density. The density (g/ml) of the coolants decreased in the order of glycerol (1.26) > water (1) > vegetable oil (0.91 – 0.93) > ethanol (0.79). As the density of hard paraffin ranges from 0.88 to 0.92 g/ml, it is plausible that the molten hard paraffin droplets float on the surface of water, glycerol and vegetable oil, but solidify in the bulk of the ethanol bath.

Although hard paraffin SLM could be fabricated using ethanol as a cooling bath, there was still the issue of the small hard paraffin patches formed on the surface of the ethanol bath. The manufacturer of the Buchi encapsulator recommended the attachment of a cooling chamber of more than 300 cm drop to solidify molten lipid droplets for the fabrication of SLM. The purchase of this cooling chamber was not feasible, neither was it possible to accommodate the housing of such a tall chamber in the laboratory. We hypothesized that the effectiveness of SLM fabrication might be improved by increasing the distance between the nozzle orifice and the coolant bath as this would allow for the enhanced partial solidification of the molten droplets before they become in contact with the coolant surface. To permit more time for the emerging lipid droplets to solidify, the distance between the nozzle orifice and the surface of the coolant bath was thus increased from 40 cm to 70 cm. This modification was observed to increase the number of hard paraffin SLM fabricated, and to reduce the size of the lipid sheets floating on the surface of the ethanol bath. To further enhance the SLM fabrication efficiency, the distance between the nozzle orifice and coolant bath was increased from 70 cm to 200 cm, and refrigerated air was circulated around the descending path of the hard paraffin liquid droplets as they fell into the coolant bath. These additional modifications were successful in limiting the formation of lipid sheets on the ethanol bath, and only spherical, well-separated SLM were produced (Figure 16).

This modified fabrication protocol was also used for the production of melatonin-loaded SLM using Witepsol H15 and Gelucire 50/13 as lipid carrier. SLM were successfully fabricated for both of these materials. However, the results were less satisfactory compared to hard paraffin SLM in that there were still small lipid sheets visible on the surface of the ethanol bath when Witepsol H15 and Gelucire 50/13 were used as lipid carriers.
Figure 15. (a) Amorphous layer produced on the surface of an iced water coolant bath placed at 40 cm below the nozzle orifice (b) Amorphous layer of hard paraffin when the coolant bath was placed at 70 cm below the nozzle. (C) Filtered amorphous layer of hard paraffin.
2.3.2 Melatonin assay development and validation

Under the specified conditions of the HPLC method, 60 μg/ml melatonin standard solution was analysed and melatonin (Figure 17a) was found to elute as a sharp peak at 8 min. Then, 1 ml of 0.1N HCl was added to 4 ml of 60 μg/ml melatonin standard solution and a forced degradation was attempted by boiling the solution for 30 s and 2 min, respectively prior to HPLC analysis (Figures 17 b and 17c). The respective chromatograms show a reduction in the peak area for melatonin, and the appearance of new peaks at about 1.7 min. The two chromatograms suggest no apparent overlaps between the peaks for the degradation products and the parent drug, indicating that the HPLC assay is stability-indicating.
Figure 17. Chromatographic peaks of melatonin before (a) and after forced degradation by boiling in 0.1N HCl for 20 s (b) and 2 min (c)
A linear calibration curve (Figure 18) was obtained for the standard melatonin solutions in the concentration range of 1 to 60 µg/ml. The precision of the assay was verified by repeatability. The intra-day and inter-day precisions were determined by analysing three different concentrations (Bioanalytical method validation 2013) (1, 20 and 60 µg/ml). Each concentration was analysed three times on three consecutive days. The result of intraday and interday accuracy and precisions are reported in Table 1.

Table 1. Precision and accuracy of HPLC assay for melatonin

<table>
<thead>
<tr>
<th>Conc of standard solution (µg/ml)</th>
<th>Intraday Mean ± SD (µg/ml)</th>
<th>Intraday Accuracy (%)</th>
<th>Intraday Coefficient of Variation (%)</th>
<th>Interday Mean ± SD (µg/ml)</th>
<th>Interday Accuracy (%)</th>
<th>Interday Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.125±0.002</td>
<td>111.6±0.60</td>
<td>0.18</td>
<td>1.064±0.058</td>
<td>106.4±5.82</td>
<td>5.45</td>
</tr>
<tr>
<td>20</td>
<td>20.800±0.002</td>
<td>104.0±0.1</td>
<td>0.01</td>
<td>20.271±0.468</td>
<td>101.3±2.34</td>
<td>2.31</td>
</tr>
<tr>
<td>60</td>
<td>61.589±0.012</td>
<td>102.6±0.2</td>
<td>0.02</td>
<td>60.347±1.096</td>
<td>100.5±1.83</td>
<td>1.82</td>
</tr>
</tbody>
</table>
Melatonin standard solutions prepared using ethanol as solvent were also quantified by UV-Vis spectrophotometry at 230 nm. There was good linearity ($R^2 > 0.99$) between the absorbance and concentration of the standard solutions in the concentration range of 0.5 to 4 µg/ml (Figure 19). The assay was therefore deemed acceptable for the quantification of melatonin leakage from the solidifying SLM into the ethanol bath during the fabrication process.

### 2.3.3 Melatonin stability

Heat had to be applied to render the lipid-melatonin mixtures into a liquid form appropriate for extrusion through the nozzle of the Buchi Encapsulator in order to fabricate the SLM. Depending on the melting point of the lipids, the temperature of the molten mixtures reached as high as 90°C. Melatonin was supplied by the manufacturer with instructions for refrigerated storage. To ensure the drug remained stable throughout the fabrication process, various melatonin samples were subjected to heat treatment at 90°C for 2h, the duration reflecting the total time from weighing the ingredients to harvesting the solidified SLM from the coolant bath. The residual drug content of these samples after heat treatment (Table 2) suggest that melatonin was a highly stable drug, and could be fabricated into SLM without loss of its chemical integrity. Even in the presence of strong acid and base, melatonin did not degrade when heated at 90°C for 2h (Figure 20). This was in contrast to the degradation observed when melatonin was boiled for 2 min with 0.1N HCl (Section 2.3.2).
Table 2. Residual melatonin content in samples subject to heat treatment at 90°C for 2 hours

<table>
<thead>
<tr>
<th>Sample</th>
<th>Residual melatonin content in sample after heat treatment (%) (mean ± SD, n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melatonin powder (5 g)</td>
<td>100.2 ± 0.03%</td>
</tr>
<tr>
<td>Melatonin standard solution (60 µg/ml in phosphate buffer, 5 ml)</td>
<td>100.8 ± 0.06%</td>
</tr>
<tr>
<td>Melatonin standard solution (4 ml) + 0.1 N HCl (1 ml)</td>
<td>102.43 ± 0.18%</td>
</tr>
<tr>
<td>Melatonin standard solution (4 ml) + 0.1 N NaOH (1 ml)</td>
<td>99.9 ± 0.10%</td>
</tr>
</tbody>
</table>

(a) and (b) graphs showing melatonin levels before and after heat treatment.
2.3.4 Characteristics of melatonin-loaded SLM

The characteristics of melatonin-loaded SLM prepared with different lipid carriers are shown in Table 3. The mean diameter of the SLM varied between 5.54 mm (Witepsol H15) and 11.01 mm (Gelucire 50/13), and was larger than the nozzle aperture size (1 mm) used for fabricating the SLM. This was because the electro-repulsive force applied on the microencapsulator to separate the liquid droplets did not work as efficiently on the non-conducting lipid materials, with the result that the molten lipid droplets tended to agglomerate as they fell towards the coolant bath. This agglomeration of lipid droplets produced spherical beads with diameters larger than the nozzle aperture.
There were significant differences in mean diameter among the three SLM (p < 0.001, one-way ANOVA). The mean size of the Gelucire 50/13 SLMs was significantly bigger than those obtained with hard paraffin and Witepsol H15 SLMs (p =0.02 and <0.001, respectively) while the hard paraffin SLM were also larger than the Witepsol H15 SLM (p < 0.001). However, the size distributions indicate considerable overlaps in the size ranges of the 3 types of SLM.

Table 3. Characteristics of melatonin-loaded solid lipid microparticles (mean ± SD for 100 microparticles)

<table>
<thead>
<tr>
<th>Lipid carrier</th>
<th>Particle diameter (mean ± SD, n = 3) (mm)</th>
<th>Size distribution (mm)</th>
<th>Melatonin encapsulation efficiency (mean ± SD, n = 3) (%)</th>
<th>Melatonin loading (mean ± SD, n = 3) (mg/g SLM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffin</td>
<td>9.56 ± 3.81</td>
<td>4.10 – 17.20</td>
<td>57.01 ± 0.03%</td>
<td>14.21 ± 0.032</td>
</tr>
<tr>
<td>Witepsol H15</td>
<td>5.54 ± 2.091</td>
<td>4.10– 14.10</td>
<td>16.21 ± 0.45%</td>
<td>4.00 ± 0.052</td>
</tr>
<tr>
<td>Gelucire 50/13</td>
<td>11.01 ± 1.861</td>
<td>6.20 – 17.90</td>
<td>20.32 ± 0.05%</td>
<td>5.00 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 21 shows the SEM micrographs of the melatonin-loaded SLMs. The hard paraffin SLM were spherical particles with relatively smooth and compact surfaces while the Witepsol H15 SLM appeared to be collapsed and irregular particles showing concavity. Gelucire 50/13 SLM were ovoid particles with rough surfaces and high porosity.
Figure 21. SEM micrographs of melatonin-loaded SLM prepared with (a) Witepsol H15®; (b) hard paraffin; and (c) Gelucire 50/13 as lipid carriers

There were significant differences in melatonin loading, with hard paraffin SLM having the highest melatonin loading, and Witepsol H15 SLM the lowest loading ($p < 0.001$). The measured melatonin content in the Witepsol H15, Gelucire 50/13 and hard paraffin SLM were 4.00, 5.00 and 14.21 mg/g, respectively. This translates into relatively low melatonin encapsulation efficiency (EE) for all three SLM, ranging from 16.2% to 57.0% with the EE increasing in the order of Witepsol H15 < Gelucire 50/13 < hard paraffin SLM. The low melatonin EE could not be readily explained by the differences in HLB and melting point values of the lipid carrier. It might, however, be related to the porosity of the SLM, as the compact hard paraffin SLM showed a 2.8 to 3.6 fold higher melatonin loading compared to the collapsed Witepsol H15 SLM and the porous Gelucire 50/13 SLM.
Melatonin content in the ethanol bath (Table 4) confirmed that a high percentage of the initial melatonin load was lost into the bath during the preparation of Witepsol H15 SLM and Gelucire 50/13 SLM. It also appeared that the smaller sized Witepsol H15 SLM was less effective than Gelucire 50/13 SLM in limiting melatonin migration from the lipid carrier into the coolant bath during manufacture. Melatonin is highly soluble in ethanol (50 mg/ml)(Melatonin 1997) and this might have accounted for the significant diffusion of melatonin from the molten lipid droplets of all three SLM into the coolant bath during fabrication.

<table>
<thead>
<tr>
<th>Lipid carrier</th>
<th>Percentage of initial melatonin load measured in the ethanol coolant bath (% w/w) (mean ± SD, n =3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffin</td>
<td>14.3± 0.01</td>
</tr>
<tr>
<td>Witepsol H15</td>
<td>70.3±0.04</td>
</tr>
<tr>
<td>Gelucire 50/13</td>
<td>57.4±0.01</td>
</tr>
</tbody>
</table>

2.4 Conclusion

SLM with melatonin loading of 4.00 to 14.21 mg/g can be successfully produced in the Buchi B-390 Encapsulator using Gelucire 50/13, hard paraffin and Witepsol H15 as lipid carriers and ethanol as coolant. The optimal distance between the nozzle aperture and coolant surface was found to be 200 cm. Although different coolants were tested, only ethanol let the lipid droplets disperse uniformly and keep their spherical shape concomitantly. The hard paraffin SLM were spherical compact particles whereas the SLM fabricated from Gelucire 50/13 and Witepsol H15 were irregularly shaped particles with higher porosity, which may have accounted for these two SLM having relatively lower melatonin loading efficiencies. The EE was low, with only 16.2 to 57.0% of the initial melatonin load found to be incorporated into the SLM.
3 Optimisation of method to fabricate melatonin-loaded solid lipid microparticles

3.1 Introduction

Preliminary experiments have shown that melatonin-loaded SLM could be successfully produced in the Buchi Encapsulator using lipids of a range of melting temperatures and ethanol as coolant. However, not all SLM fabricated were spherical particles with compact surfaces, and the melatonin loading efficiency was relatively low. It was hypothesized that the method employed for cooling the molten lipid droplets was not ideal, and that an alternative coolant should be used. Ethanol is a co-solvent commonly employed to enhance the dissolution of poorly water-soluble drugs. Its use as a coolant appeared to promote melatonin dissolution and result in a high percent of the melatonin load migrating from the SLM into the coolant bath.

Two alternative coolants were considered to further optimise the fabrication process. We first trialled solid carbon dioxide (\(\sim -80^\circ\text{C}\)) as the coolant; however, the molten lipid droplets were unable to retain their spherical shape upon impact with the solid carbon dioxide, and an agglomerated mass was produced for all three types of SLM. The second coolant employed was liquid nitrogen (\(\sim -196^\circ\text{C}\)), and the data obtained for this coolant is reported in this section of the study.

Nitrogen at atmospheric conditions is a colourless, odourless, tasteless, non-irritating, and inert gas. Nitrogen compressed at high pressure into liquid nitrogen is a highly effective coolant employed for a wide range of applications in the food and pharmaceutical industries. With regards to SLM fabrication, liquid nitrogen has been used to freeze particles or pellets produced by molten wax granulation, and the resultant particles were found to be spherical and nonporous (Cheboyina et al. 2004). Using liquid nitrogen as a cooling bath in the fabrication of SLM is a novel and simple technique that offers several advantages over other coolants. Liquid nitrogen does not only provide safe, efficient and environmentally friendly freezing, it is also able to improve product yield, optimize equipment performance, protect product quality, and make manufacturing operations safer (Cheboyina, Chambliss & Wyandt 2004). In this study, the extruded lipid droplets were allowed to fall into a liquid nitrogen bath (-196° C), and the solidified particles were collected after the complete evaporation of liquid nitrogen at ambient temperature.
3.2 Materials and Methods

3.2.1 Materials

Liquid nitrogen was purchased from BOC Ltd (Perth, Australia). All other materials were the same as those listed in Section 1.2.1.

3.2.2 Preparation of melatonin-loaded SLM

Melatonin-loaded SLM were fabricated using the Buchi Encapsulator operating under the same conditions as those described in Section 2.2.2. The melatonin-lipid mixtures for extrusion into droplets were also prepared in the same manner as those described in Section 2.2.2. The extruded molten lipid droplets were allowed to fall into a coolant bath containing 1 L of liquid nitrogen in a 3 L dewar flask. The distance between the nozzle aperture and the coolant surface was 50 cm. Solidified SLM were collected from the bath after the liquid nitrogen had vaporised to dryness at ambient temperature. Dried SLM were stored in glass containers at ambient conditions until they were analysed. All analyses were performed within one month of manufacture of the SLM.

3.2.3 Characterization of the SLM

3.2.3.1 Particle size analysis

Particle size analysis was performed according the method described in Section 2.2.3.1.

3.2.3.2 Morphological Analysis

Morphological analyses of the SLMs were performed according to the method described in Section 2.2.3.2.

3.2.3.3 Differential scanning calorimetry (DSC)

Physical changes and drug-lipid interactions were determined using differential scanning calorimetry (DSC). Material phase transitions, such as melting, glass transition, exothermic decompositions, and interactions with other materials, which involve energy changes or heat capacity changes can be detected by DSC with great sensitivity (Chiu & Prenner 2011).
The DSC analysis of the melatonin-loaded SLM was performed using a TA DSC Q10 series equipment TA instruments, C/O Waters Australia Pty. Ltd (Rydalmere NSW 2116, Australia) with nitrogen as a purge gas. Each sample was weighed (15±1 mg) onto an aluminium sample pan, crimped with aluminium cover and analysed over the temperature range of -20°C to 150°C at a heating rate of 10°C/min.

3.2.3.4 Assay of melatonin content

Assays of melatonin content in the SLM were performed according to the methods described in Section 2.2.3.4.

3.2.3.5 In vitro drug dissolution studies

In vitro drug dissolution profiles were determined for free melatonin and melatonin-loaded SLM using a USP paddle apparatus VARIAN VK 7010, Agilent (Mulgrave, Victoria 3170, Australia) operating at 50 rpm. The dissolution medium comprised of 500 ml of 0.1 M HCl at 37 °C. Samples, each weighing 400 mg, were drawn from triplicate batches of melatonin-loaded SLM or from free melatonin in the dissolution experiments. Aliquots of 1 ml were withdrawn from the dissolution medium at 5, 10, 15, 20, 30, 40 and 60 min for assay of melatonin content using the established HPLC method. The cumulative percentage release of melatonin load was expressed as a function of time to give the respective drug dissolution profiles.

3.3 Results and Discussion

The use of liquid nitrogen as coolant enabled the successful fabrication of melatonin-loaded SLMs using Gelucire 50/13, Witepsol H15 and hard paraffin as the lipid carriers. Each batch of SLMs was fabricated using the Buchi Encapsulator with 97.5 g of lipid, 2.5 g of melatonin, a nozzle orifice diameter of 1 mm, and 1 L of liquid nitrogen as coolant. The distance between the nozzle orifice and the surface of the liquid nitrogen bath was 50 cm. The extremely low temperature of the liquid nitrogen bath caused the liquid lipid droplets to solidify instantaneously on contact. When the required weight of molten melatonin-lipid mixture had been extruded, they were allowed to remain in the liquid nitrogen bath until the complete evaporation of the liquid nitrogen (approximately 5 min). The solidified lipid particles were readily retrievable as intact particles with no apparent agglomeration. For all three types of SLMs, the majority of the particles were spherical in shape.

There was no significant change in the diameter of the melatonin-loaded Gelucire 50/13 and hard paraffin microparticles switching from an ethanol coolant bath (Table 5) to a liquid nitrogen coolant
bath (Table 5) ($p > 0.05$), however, Witepsol H15 microparticles cooled in liquid nitrogen are significantly larger than Witepsol H15 microparticles cooled in an ethanol bath ($p < 0.0005$).

ONE-WAY ANOVA analysis shows a significant difference in particle sizes of the three melatonin-loaded SLM (Table 5, $p < 0.0005$). Fisher’s Least Significant Difference Post hoc test indicates that Gelucire 50/13 particles are bigger in size than hard paraffin particles ($p < 0.0005$), however there is not a significant difference between the particle sizes of hard paraffin and Witepsol H15 microparticles ($p > 0.05$). It is also shown that Gelucire 50/13 SLM are significantly bigger than Witepsol H15 SLM ($p < 0.0005$).

While there was no change in the diameter of the SLM, there was a marked improvement in the melatonin EE when the coolant was switched from ethanol to liquid nitrogen (Table 5). SLM fabricated using liquid nitrogen as the coolant showed mean EE of above 84%. Of the three melatonin-loaded SLMs, the hard paraffin SLM had the highest EE of 90% while the Witepsol H15 SLM had the lowest EE of 84.4%. The EE values were significantly different amongst the three types of SLMs ($p < 0.0003$, one-way ANOVA).

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>Mean size</th>
<th>Size distribution</th>
<th>EE%</th>
<th>Melatonin loading (mg/g SLM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffin</td>
<td>10.11±3.48 mm</td>
<td>6.70-20.30 mm</td>
<td>90.03±0.06%</td>
<td>22.50±0.20 mg</td>
</tr>
<tr>
<td>Witepsol H15</td>
<td>6.77±2.44 mm</td>
<td>1.00-12.20 mm</td>
<td>84.41±0.22%</td>
<td>21.10±0.30 mg</td>
</tr>
<tr>
<td>Gelucire 50/13</td>
<td>11.08±2.77 mm</td>
<td>8.10-15.40 mm</td>
<td>88.12±0.02%</td>
<td>22.00±0.20 mg</td>
</tr>
</tbody>
</table>

Figure 22 shows the SEM images of the melatonin-loaded microparticles. Unlike the SLM fabricated using ethanol as coolant, the SLM fabricated using liquid nitrogen as coolant were mainly spherical microparticles with compact, non-porous surfaces. Moreover, there was little apparent difference in surface morphology amongst the melatonin-loaded SLM fabricated with Gelucire 50/13, hard paraffin and Witepsol H15.
Figure 22. SEM micrographs of melatonin loaded SLM fabricated using liquid nitrogen as coolant and (a) Gelucire 50/13; (b) hard paraffin; and (c) Witepsol H15 as lipid carrier
3.3.1 In vitro drug release profile

The drug dissolution behaviour of the three types of microparticles is shown in Figure 23. Despite their similar particle morphology and high EE, the three types of SLM exhibited vastly different drug release profiles.

Melatonin dissolution rate was faster from the Gelucire 50/13 SLM than that of the unencapsulated melatonin. At 20 min, 92% of the loaded melatonin was released from Gelucire 50/13 SLM, compared with 68% from the melatonin powder ($p < 0.0005$). At 15 min, more than 90% of the melatonin load was released from the Gelucire 50/13 SLM while the unencapsulated melatonin achieved a dissolution rate of 66% ($p < 0.0005$). At 60 min, the release of melatonin from Gelucire 50/13 microparticles was 26.6% more than from the melatonin powder ($p < 0.0005$).

Melatonin release from the Witepsol H15 SLM was slow initially, but the rate increased dramatically after 5 min, and was similar to that in the unencapsulated melatonin at 15 min (66.9% vs. 66%). At 60 min, the release of melatonin in Witepsol H15 microparticles surpassed that of the melatonin powder by 13.1% ($p < 0.05$).

Conversely, the hard paraffin SLM showed a slow and limiting melatonin release profile. At 15 min, only 12.4% of the melatonin load was released from this SLM, which increased to 19.2 % at 60 min. Prolongation of the dissolution test to 180 min did not result in significantly more melatonin released from the hard paraffin SLM (Figure 24).

The differences in drug dissolution profiles might be explained by the nature of the solid lipid carrier. Hard paraffin SLM recovered from the dissolution medium at 60 min did not show any apparent changes in surface morphology, whereas no particles were found in the dissolution media for the Witepsol H15 and Gelucire 50/13 SLM after 60 min. Gelucire 50/13 is a non-ionic water-soluble surfactant. The rapid initial dissolution profile of the Gelucire 50/13 SLM, similar to that seen for unencapsulated melatonin, suggests that the Gelucire 50/13 SLM had dissolved readily in the dissolution medium to disperse the melatonin load. The improved dissolution profile of Gelucire 50/13 SLM compared to the unencapsulated melatonin could be attributed to the effectiveness of the surface active Gelucire 50/13 in enhancing the wettability and solubility of the dispersed melatonin load in the dissolution medium.

Witepsol H15 has a melting temperature range of 37-60 °C (Figure 25c), and will therefore melt in the dissolution medium. The initial melatonin dissolution rate from Witepsol H15 SLM was slower than the unencapsulated melatonin, suggesting that the melting process did not occur instantaneously but might have required at least 5 min to reach completion. Once the melatonin
load was dispersed, the dissolution rate of the SLM caught up with the unencapsulated melatonin powder, and then surpassed it. Like Gelucire 50/13, Witepsol H15 also contains surface active compounds (triglycerides) capable of enhancing the wetting and solubility of melatonin in aqueous media.

Hard paraffin, on the other hand, is comprised of hydrocarbons that have a melting range of 40 - 60 °C (Figure 25b). Thus, the hard paraffin SLM would neither dissolve nor melt in the dissolution medium, and the intact particles were retrievable from the dissolution medium even after 180 min. The 19.2% melatonin load released from hard paraffin SLM into the dissolution medium is probably reflective of melatonin present on/near the SLM surface that was accessible for dissolution by the aqueous medium.

![Release Chart](image)

**Figure 23.** In vitro drug release profile of melatonin-loaded SLM incubated in 500 ml of 0.1 M HCl at 37 °C over the course of 60 min
3.3.2 Thermal analysis

DSC thermograms for melatonin, the respective lipids, physical mixtures of melatonin and the lipids, and the three different types of melatonin-loaded SLM are shown in Figure 25. Melatonin and Witepsol H15 displayed relatively sharp melting endotherms with a peak temperature of 120 °C and 40 °C, respectively. Gelucire 50/13 and hard paraffin exhibited broader melting endotherms characterized by multiple peaks in the temperature ranges of 37-60 °C and 40-60 °C, respectively. Peaks attributable to the drug and lipid were retained when melatonin was physically mixed with Gelucire 50/13 or Witepsol H15. The physical mixture of melatonin and hard paraffin showed the melting endotherm for the lipid, but the endotherm for the melatonin had shifted significantly to a lower peak temperature of 108°C, and there was a marked decrease in peak area for the drug. An integration of the peak areas suggest a recovery of 38.8% of melatonin for the hard paraffin and melatonin mixture, compared to 98.4 and 97.8% recovery for the Gelucire 50/13/melatonin and Witepsol H15/melatonin physical mixtures. This indicates a potential interaction of the melatonin powder with the melted hard paraffin.

Thermograms for the SLM show retention of the lipid and melatonin peaks for the Gelucire 50/13 SLM. Peak area measurements suggest a recovery of 98.4% and 106.1% of the melatonin and
Gelucire 50/13 loads, indicating a lack of interaction between Gelucire 50/13 and melatonin during the SLM fabrication process, and the presence of crystalline melatonin and Gelucire 50/13 in the SLM. In the case of Witepsol H15 SLM, there was retention of the Witepsol H15 peak with recovery of 98%, but the melatonin endotherm had significantly reduced peak area, the recovery being 53%. As about 80% of the drug load was available for release in 60 min from the Witepsol H15 SLM, this reduction in melting enthalpy is not likely due to lipid-melatonin interaction, but the presence of amorphous drug in the Witepsol H15 SLM. The amorphous drug load would also correlate with the higher melatonin dissolution rate obtained for Witepsol H15 SLM compared to the unencapsulated crystalline melatonin. No melatonin endotherm was apparent for the hard paraffin SLM, while the melting endotherm for the lipid showed changes in peak characteristics. This is suggestive of interaction between hard paraffin and the drug, which may further explain the unavailability of the melatonin load for release from the hard paraffin SLM in the dissolution experiments.
Figure 25. DSC thermograms of melatonin, lipid, physical mixtures of melatonin and lipid and melatonin-loaded SLM for (a) Gelucire 50/13; (b) hard paraffin; and (c) Witepsol H15
Table 6. DSC results of physical mixtures of melatonin with three waxes and melatonin-loaded SLM

<table>
<thead>
<tr>
<th>Sample</th>
<th>melatonin content based on weight of sample used for DSC analysis (mg) (mean ± SD, n = 3)</th>
<th>melatonin content based on peak area of melting endotherm (mg) (mean ± SD, n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical mixtures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hard paraffin + Melatonin</td>
<td>5.00</td>
<td>1.96 ± 0.04</td>
</tr>
<tr>
<td>Gelucire 50/13 + Melatonin</td>
<td>5.00</td>
<td>4.92 ± 0.02</td>
</tr>
<tr>
<td>Witepsol H15 + Melatonin</td>
<td>5.00</td>
<td>4.89 ± 0.03</td>
</tr>
<tr>
<td>Melatonin-loaded SLM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hard paraffin SLM</td>
<td>0.34</td>
<td>-</td>
</tr>
<tr>
<td>Gelucire 50/13 SLM</td>
<td>0.33</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>Witepsol H15 SLM</td>
<td>0.32</td>
<td>0.17 ± 0.04</td>
</tr>
</tbody>
</table>

3.4 Conclusion

Spherical and compact SLM with melatonin loading of 21.1 to 22.4 mg/g can be successfully produced in the Buchi B-390 Encapsulator using Gelucire 50/13, hard paraffin and Witepsol H15 as lipid carriers and liquid nitrogen as coolant, and with the distance between the nozzle aperture and coolant surface at 50 cm. In vitro dissolution profiles showed rapid and complete release of the drug load from the Gelucire 50/13 SLM, and a slightly delayed but also near complete release of melatonin from the Witepsol H15 SLM. Both SLM provide higher rates of drug dissolution in 0.1 M HCl compared to unencapsulated melatonin. Only 19% of the melatonin load in the hard paraffin SLM was released into 0.1 M HCl after 60 min; the poor drug availability is attributable to drug-lipid interaction during the fabrication process.
4 Sustained-release melatonin-loaded solid lipid microparticles

4.1 Introduction

In Section 3, it was shown that melatonin-loaded SLM could be successfully produced in the Buchi Encapsulator using lipids of low melting points. In vitro dissolution profiles of the Gelucire 50/13 SLM and Witepsol H15 SLM indicate that these are immediate release products capable of releasing >75% of the melatonin load within 60 min in 0.1M HCl. The hard paraffin SLM had a much slower rate of melatonin release; however, it did not qualify to be a sustained release melatonin product because there was a plateauing of drug release at about 40 min, and the majority of the melatonin load (>80%) failed to be released even with prolonged incubation of the SLM in the dissolution medium.

In this part of the research, the aim was to produce sustained-release melatonin-loaded solid lipid microparticles that would release therapeutic levels of melatonin over a prolonged period to prevent nocturnal awakening in patients with primary insomnia and to allow them to stay asleep overnight.

Carnauba wax is extracted from the leaves of the palm Copernicia prunifera, a plant native and endemic to the north eastern Brazilian states. It is known as "queen of waxes" and in its pure state usually comes in the form of hard yellow-brown flakes. Carnauba wax consists of fatty acid esters (80-85%), fatty alcohols (10-16%), acids (3-6%) and hydrocarbons (1-3%). Carnauba wax has a very high melting point of 82-86 °C. It is practically insoluble in water and ethanol, but soluble in heated ethyl acetate and in xylene. It is non-toxic and hypoallergenic. Its density is 0.97 g/cm³. Carnauba wax is widely used to modify the release of active ingredients in pharmaceutical industry (Nart et al. 2017); to this end it has also been chosen to control the release of melatonin (Albertini et al. 2014).

4.2 Materials and methods

4.2.1 Materials

Carnauba wax and stearic acid were purchased from PharmAust Manufacturing PTY LTD (Malaga Western Australia 6090, Australia). All other materials are the same as those described in Section 2.2.1.
4.2.2 Preparation of microparticles

The goal was to produce 2.5% w/w melatonin-loaded sustained release SLMs with the Buchi-390 Encapsulator using carnauba wax as lipid carrier. A total weight of 100 g of the mixture was used to prepare a batch of SLM.

2.5 g Melatonin and 97.5 g carnauba wax were heated to 90 °C. The molten mixture was then loaded into the feeding chamber of the Buchi encapsulator for extrusion. The inlet air pressure was set at 2 bars.

To avoid the solidification of the molten drug-lipid mixture, the feeding tube of the encapsulator was heated and maintained at a temperature of 90 °C by wrapping a heating element SDC240JC-A, Briskheat®(Columbus, Ohio, USA) around the tube. To protect the plastic components of the encapsulator from being damaged by the high temperature of the heating element, the plastic components were insulated with felt cloth. The experimental set up is shown in Figure 26.
Figure 26. Preparation of Buchi Encapsulator for the fabrication of melatonin-loaded carnauba wax SLM. Feeding tube was maintained at 90°C by wrapping with a heating element, to avoid solidification of the molten lipid. Plastic components of the encapsulator were insulated from the heat using orange felt cloths

4.3 Result and Discussion

As the melting temperature of carnauba wax was 82-86°C, the feeding tubes and nozzle of the encapsulator had to be maintained at a temperature at 90°C to avoid solidification of the melatonin-lipid mixture during fabrication. It was possible to keep the drug-lipid mixture in a liquid form in the feeding bottle by heating on a hot plate and in the feeding tube by wrapping the tube with a heating element. However, we were unable to find a way to increase the temperature of the encapsulator nozzle to 90°C. The encapsulator provided for the heating of the nozzle head to 80°C, but this was found inadequate to maintain the extruding melatonin-lipid mixture in liquid form. Immediately
after entering the nozzle, the molten melatonin-lipid mixture would solidify, eventually blocking the nozzle and preventing further extrusion of the molten liquid. After numerous unsuccessful attempts to resolve this issue, it was concluded that the Buchi Encapsulator in its current form in the laboratory was not designed for fabricating microparticles with solid lipids with melting temperatures above 70°C.

4.4 Conclusion

Although carnauba wax is extensively used in pharmaceutical industry to control the release of active ingredients, its high melting temperature made it unsuitable in the employed methodology to produce sustained-release microparticles.

In spite of trying a variety of modifications to the apparatus (e.g. using a wrapped heater stirrer and heating tube all the way from the pressurized bottle to the feeding tube and the nozzle to maintain the temperature 10°C above the melting point of carnauba wax), we could not produce microparticles. Carnauba wax would solidify as soon as it reached the nozzle as the maximum temperature set for the nozzle was 80°C which is less than the melting temperature of Carnauba wax is.
5 Final Conclusion

Melatonin is a versatile molecule produced primarily in the pineal gland in the brain. It is synthesized from the amino acid tryptophan and the neurotransmitter serotonin and is a chief regulator of sleep. Melatonin is used in the geriatric population to modify their sleep pattern. There is also growing evidence that it is widely used in children with neurodevelopment disorders who suffer from lack of sleep due to low blood melatonin levels (Bunn 2013).

There are different melatonin formulations in the market as a dietary supplement or prescribed medicine for the geriatric population. Although immediate release formulations help patients fall asleep, bioavailability of melatonin is low and variable. In addition, due to melatonin’s high first pass metabolism, the concentration of melatonin in the blood drops to subtherapeutic levels in less than 2 hours after administration. Hence, patients taking these formulations can suffer from nocturnal awakening. To solve this issue, sustained release formulations are suitable to provide a constant drug release over a period of eight hours to help patients stay asleep during the night, however, these formulations do not provide a high enough initial dose to help patients fall asleep. Therefore, this project aimed to develop a formulation with a combination of immediate and sustained release particles, which was hoped to not only help patients fall asleep but also to help them stay asleep throughout the night.

Immediate release lipid microparticles, aimed to increase the bioavailability of melatonin by increasing dissolution, were successfully produced by the Buchi Encapsulator using three different lipids. However, when using an ethanol cooling bath to solidify the molten microparticles, ethanol dissolved the melatonin loaded in these SLM and therefore the melatonin encapsulation efficiency for all three produced microparticles was not acceptable.

When liquid nitrogen was chosen as a coolant, melatonin remained entrapped in the produced microparticles and all three SLM showed very good encapsulation efficacy. The highest EE% with 90% was seen with hard paraffin. However, it seems that while hard paraffin effectively entrapped melatonin, it did not allow its effective in vitro dissolution. Hence, melatonin released from hard paraffin microparticles was less than 20% which was deemed unacceptable.

Melatonin loaded Gelucire 50/13 microparticles with encapsulation efficacy of 88% were found to have the best release profile when compared to Witepsol H15 and hard paraffin. It released more than 90% of the active ingredient within 15 min. Gelucire 50/13 also increased the dissolution of its melatonin load in comparison to pure melatonin powder, which dissolved by 26.6% in 15 min.

Regarding the attempted manufacture of sustained release microparticles, carnauba wax was chosen as lipid carrier as it is commonly used to modify the release of active ingredients in the
pharmaceutical industry. Carnauba wax with a melting temperature of 82-86 °C solidified immediately in the connecting tube to the nozzle. Although the solidification issue in the tubes connecting the pressure bottle to the nozzle could be resolved by installing a heating tube, the problem persisted in the nozzle itself. Regrettably the temperature in the nozzle, which is designed to withstand a maximum temperature of 80 °C, could not be increased sufficiently to melt carnauba wax.

As the Buchi Encapsulator is not designed for high melting point lipids such as carnauba wax, future research should test alternative lipid carriers with lower melting points which can modify the release of melatonin and can also be used in the Buchi Encapsulator to prepare microparticles.
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