Formulating curcumin in a biodegradable polymeric composite material: A step towards wound healing applications

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made. The thesis also contains no material which has been accepted for the award of any other degree or diploma in any university. Also, this thesis does not exceed 50,000 words and complies with the provisions set out for the degree of Master of Philosophy by the University of Melbourne.

Maryam Shahnia

March 2017
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Dedication

To my beloved Mother and Father,

for their unconditional love, encouragement and support in my entire life
Abstract

The natural process of wound healing typically consists of four distinct but overlapping phases which include, hemostasis (platelet aggregation and blood clot formation), inflammation (migration of blood cells), proliferation (angiogenesis or blood vessel formation), and remodelling (reorganisation of collagen and scar tissue formation). However, in diabetic patients, this elaborate well-programmed process becomes disrupted, and there is an urgent need for compounds and formulations that can improve wound healing in these cases. A variety of natural components, including curcumin, have been identified as wound-healing agents. Curcumin, is a yellow hydrophobic natural polyphenolic pigment derived from the rhizomes of the herb *Carcuma longa*, which has been identified as the active principal of turmeric. The inability to efficiently deliver curcumin in a soluble form presents a chief challenge for its clinical use. Here we characterised, and optimised different biodegradable and biocompatible formulations of curcumin encapsulated particles, in order to enhance the efficiency of curcumin wound healing effect.

The size of the optimised curcumin particles ranged from 1286 to 1485 nm, with an encapsulation efficiency of 75%. The zeta potential exhibited values in the range of (-7.2) to (-7.96) with the PDI of 0.4. Physical characterisation using TEM imaging ensured the successful fabrication and encapsulation of curcumin in the polymeric matrix, which had been fabricated in rod shape. Release profile occurred in a biphasic manner including an initial burst, followed by a sustained release trend for curcumin particles. *In vitro* cytotoxicity assays along with microscopic imaging confirmed safety of the applied concentration of curcumin particles below 25 µg/ml. Moreover, the results of cellular uptake study validated the internalisation of curcumin particles. Overall this thesis, elucidated the developed biocompatible and biodegradable formulations for curcumin
encapsulation do have the potential to be employed as a drug delivery vehicle for curcumin. Further validation of the potential of this preparation to enhance wound healing is still needed.
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Chapter 1: Introduction

This chapter presents a literature review on curcumin properties and its application as a valuable bioactive in drug delivery and pharmaceutical sciences, and the recent investigations, which established numerous approaches to improving the limitations of curcumin application in the mentioned fields. This chapter further outlines the hypotheses, the existing gaps in the application of curcumin as a wound healing agent, and aims of this research.

1.1. Turmeric and its active ingredients

The isolation of antibiotic-resistant pathogens, coupled with increased awareness of people about the side effects of synthetic drugs have resulted in the search for different formulations of natural medicinal plants.

One of these highly impressive medicinal plants is turmeric, which belongs to the Zingiberaceae family with the scientific name of *Curcuma longa* L. (Goel et al. 2008). Turmeric has other names such as Jiang Huang, harida, and Indian saffron and obtained from the rhizomes of the plant. The history of turmeric dates back to 3000 BC in Asia where people had used an ointment based on it for relieving food poisoning effects (Anand et al. 2008). It has been used for centuries in the Ayurvedic system of medicine as a blood purifier (Aggarwal et al. 2003). Turmeric has been used in home remedies for the treatment of skin diseases and softening of rough skins in the shape of bath soaps and creams in India. Furthermore, the curing effects of turmeric on cuts, bruises and wounds have been known
over centuries. Turmeric was introduced to Europe by Arab traders in the 13th century and its use as an exceptional herb attracted the spotlight of Westerners due to its broad spectrum of medicinal advantages. Turmeric consists of 3-5% curcuminoids, which include curcumin (deferuloyl methane), as the most important fraction, desmethoxycurcumin, and bisdemethoxycurcumin. Among these components, curcumin serves a great role in biological and therapeutic activities of turmeric. Figure 1 shows turmeric along with the chemical structure of the three curcuminoids (Aggarwal et al. 2003, Goel et al. 2008).

1.2. Curcumin

Curcumin is a hydrophobic component which was isolated in 1815 for the first time, but after around 158 years the chemical structure was determined in 1973 by Roughley and Whiting (Roughley and Whiting 1973). The chemical structure of curcumin consists of two rings, both of which have a methoxy ether group in the ortho position.

Curcumin is soluble in ethyl alcohol, propylene glycol and glacial acetic acid but insoluble in water. The maximum absorbance of curcumin is at 425 nm. Curcumin has been acknowledged as a nontoxic chemical for humans as a result of many studies (Shoba et al. 1998, Qureshi et al. 1992). Curcumin is responsible for the yellow colour of turmeric. The melting point of curcumin is 176-177°C, and it makes a reddish-brown salt with alkali (Braga et al. 2003).
Figure 1. a: Turmeric plant, b: Turmeric rhizome, c: Turmeric powder (Palve and Nayak 2012), and d: Chemical structure of the three curcuminoids (Aggarwal et al. 2003)
1.2.1. Pharmacological activity of curcumin

According to many clinical and preclinical studies conducted on curcumin during the past decades, curcumin has been broadly acknowledged as a “wonder drug of the future”, due to its extraordinary potential in preventing and treating several incurable illnesses (Epstein et al. 2010).

Over the last 50 years, curcumin has been investigated for its anticancer properties. These studies included the effect of curcumin on breast cancer (Carroll et al. 2008), colon cancer (Shpitz et al. 2007, Moos et al. 2004), stomach cancer (Ikezaki et al. 2000), liver cancer (Chuang et al. 2000), and oral cancer (Azuine and Bhide 1992) for which curcumin showed chemotherapeutic effects.

Other studies included the effects of curcumin on diseases such as Alzheimer (Pan et al. 2008), gastric ulcer (Prucksunand et al. 2001), rheumatoid arthritis (Dcodhar et al. 2013), diabetes (Usharani et al. 2008), psoriasis (Heng et al. 2000), bowel syndrome (Bundy et al. 2004) and HIV (Balasubramanyam et al. 2004), which revealed the potential of curcumin as a useful therapeutic agent.

Further studies have investigated the effects of curcumin on the cardiovascular system, as well as pulmonary and metabolic diseases (Aggarwal and Harikumar 2009). Also, studies indicated the antimicrobial (Basniwal et al. 2011), antiviral (Zandi et al. 2010), antioxidant (Binion et al. 2008), anti-inflammatory (Liang et al. 2009) and wound healing effects of curcumin extensively (Panchatcharam et al. 2006, Sidhu et al. 1998).
1.2.2. Limitations of curcumin application as a bioactive agent

An ideal drug delivery system must be safe and deliver the encapsulated compound efficiently at the site of action without exhibiting any adverse effects. Another central aspect of drug delivery which should be taken into consideration is the release properties of the system, which should happen in a desirable manner in order to show effective impacts (Soppimath et al. 2001).

A large number of studies have investigated the biological and pharmacokinetic characteristics of curcumin in animals, and to a lesser extent in humans (Epstein et al. 2010, Ghalandarlaki et al. 2014). The majority of which have indicated that the poor solubility of curcumin in water has restricted its full potential because it restricts bioavailability, stability, and gastrointestinal absorption. Therefore, a wide range of research have been conducted to improve the pharmacokinetic properties of curcumin in the past decades by developing various formulations and structures to convey it efficiently to the targets (Ghalandarlaki et al. 2014).

Different structures have been used for curcumin delivery to date which include:

1.2.3. Micro- and nanoparticles

Nano- and microparticles are small particles in the range of nanometer and micrometre respectively including the core and the membrane as the main parts. The most significant particularity of micro- and nanoparticles is their remarkably small size which effectively increases the surface area to volume ratio, enabling them to penetrate the target efficiently (Brannon-Peppas 1995).

Nano- and microparticulate systems for drug delivery have made a tremendous advance towards enhancing bioavailability of hydrophobic drugs. These systems have given scientists
an opportunity to deliver the drugs specifically to the targeted sites, and overcome poor solubility, bioavailability, inefficient permeability, rapid elimination and extensive metabolism. They also protect the drug from migrating away from the target sites or being cleared out of the body through biological degradation, as well as decreasing the required dose due to their ability to maintain the applied quantity through proper encapsulation (Brannon-Peppas 1995).

Micro- and nanoparticles have been extensively used in different clinical and non-clinical trials in sustained and controlled patterns. Typically, drugs are trapped inside the particles or adsorbed to the particle surface. Drug release from the particulate system generally is specified with an initial burst release attributed to the drug adsorbed on the particle surface, followed by a sustained and controlled release featuring the release of the trapped drug due to particle degradation (Brannon-Peppas 1995).

1.2.4. Liposomes

Liposomes are artificial and synthetic vesicles described for the first time in 1965, with a globular character that are formed of an aqueous core and amphiphilic lipid bilayer and classified based on their lamellarity and size. Small unilamellar vesicles (SUV) are of 20-100 nm size, while large unilamellar vesicles (LUV) having a size of greater than 100 nm and large multilamellar vesicles (MLV) having a size of greater than 0.5 µm (Faraji and Wipf 2009, Cho et al. 2008). They are stated to behave as immunological adjuvants and drug carriers (Aqil et al. 2013). Liposomes can be applied for encapsulation of drugs with broadly varying solubility or lipophilicity entrapped either in the aqueous core of the phospholipid bilayer or at the bilayer interface (Aqil et al. 2013). In addition, they are able to deliver drugs
into cells by fusion or endocytosis, and practically any drug regardless of its solubility can be entrapped in liposomes (Aqil et al. 2013) (Figure 2).

**Figure 2.** Curcumin encapsulated in liposome and the mechanism of entrance. Adapted from (Ghalandarlaki et al. 2014) under creative commons rules

### 1.2.5. Micelles

Micelles are nanosized vesicular membranes which become soluble in water by gathering the hydrophilic heads outside in contact with the solvent and hydrophobic tails inside, which is known as emulsification (Aqil et al. 2013). They arrange themselves in a spherical form in aqueous solutions with a very narrow range of 10 to 100 nm in size which increases their stability in biological fluids. The functional properties of micelles are based on amphiphilic block copolymers, which provides a nanosized core or shell structure in the aqueous media (Jones and Leroux 1999). The hydrophobic core area stays as a pool for hydrophobic drugs and is stabilised by the hydrophilic shell. Polymeric micelles act as transporters of water-
insoluble drugs such as curcumin, which can increase the drug’s efficiency by targeting
definite cells or organs leading to accumulation of fewer drugs in healthy tissues and
consequently decreased toxicity. Therefore, occasionally higher doses can be administered
(Jones and Leroux 1999).

1.2.6. Niosomes

Niosomes are microscopic vesicular constructions that are formed by self-association of
nonionic surfactants of alkyl or dialkyl polyglycerol ether category with cholesterol, which
were first established in the 70s (Cho et al. 2008). Niosomes can provide a container for drug
molecules with wide solubility range owing to the presence of amphiphilic, hydrophilic and
lipophilic moieties in their constitution (Azmin et al. 1985). Their architecture is similar to
liposomes in vivo and can be applied as an effective alternative to liposomal drug carriers,
because of their good stability, low cost, and ease of storage for different industries including
pharmaceutical, cosmetic and food applications (Azmin et al. 1985). Niosomes can be
applied in a number of potential pharmaceutical and therapeutic applications, such as
anticancer and anti-infective drug targeting agents (Aqil et al. 2013). They have the capacity
to improve the therapeutic indices of drugs by restricting their action on the target cells. They
also improve oral bioavailability of drugs which are poorly absorbed such as curcumin to
design a potential drug delivery system and boost the skin penetration of drugs (Jain et al.
2005).
1.2.7. Cyclodextrins

Cyclodextrins (Cds) are a group of complexes created from sugar molecules bound together in cyclic oligosaccharides (Menuel et al. 2007). Cds are produced from starch by using an enzymatic switch and are generally used in agriculture and in environmental engineering in food, drug delivery systems, and chemical industries (Menuel et al. 2007, Davis and Brewster 2004). Cds form water-soluble inclusion complexes with small molecules and portions with large complexes (Davis and Brewster 2004, Menuel et al. 2007). Cds consist of an interior hydrophobic surface, which can provide a place for the residence of poorly water-soluble molecules and an external hydrophilic area, which makes its solubility possible in the aqueous setting. Additionally, Cds can augment bioavailability of insoluble drugs such as curcumin by rising drug solubility and dissolution. Cds are also able to increase the permeability of hydrophobic agents by making them accessible at the surface of the membrane’s biological barrier (Aqil et al. 2013). According to the literature, Cds help improve, the hydrolytic stability of curcumin, the protection against decomposition, the bioavailability, and the molecular dispersion compared to the free curcumin without altering their pharmacokinetic characteristics (Tomren et al. 2007, Yadav et al. 2010, Darandale and Vavia 2013) (Figure 3).
1.2.8. Dendrimers

Dendrimers were introduced in the mid-1980s, and are referred to as synthetic proteins, which are created with structural controls that match traditional biomolecules. They are a group of greatly split globular polymers which have different chemical and surface-related properties. They have controlled structures with a globular shape with much higher accuracy. They also have a single molecular weight instead of a distribution of molecular weights
typically observed in traditional linear polymers (Namazi and Adeli 2005). The dendrimer structures are consisted of a core, branched interiors, and numerous groups of surface functionals and act as a platform allowing extra substrates to be added in a highly controlled way (Longmire et al. 2008). This nano space acts as a separate environment, which decreases the payload related toxicity. The exceptional structure, dense spherical form controllable surface, monodispersity and size properties of dendrimers result in outstanding characteristics for drug delivery applications. Furthermore, the biocompatibility of dendrimers increases their effectiveness in molecular imaging, which can be enlarged by functionalization with small molecules of lower generation branch cells with anionic or neutral groups rather than similar branch cells of higher generations that have groups of the cationic surface. There are studies suggesting a potential use for them in curcumin delivery (Yallapu et al. 2011, Sarbolouki et al. 2012, Alizadeh et al. 2012, Babaei et al. 2012).

1.2.9. Nanogels

Nanogels are a group of three-dimensional cross-linked polymer networks, which are created by the help of covalent linkages and can also be modified to gel networks with degradable and biocompatible properties. The porosity among these cross-linked networks not only serves as a perfect reservoir for loading drugs but also keeps them from environmental degradation. The swelling property of nanogels is controlled in an aqueous environment by using the polymer chemical structure, cross-linking degree and the polyelectrolyte gel’s charge density and/or by pH level, ionic strength, as well as the chemical nature of the low molecular mass (Yallapu et al. 2011). Besides, nanogels can be altered chemically to include different ligands for delivery of the targeted drug, the release of the triggered drug, or
composite materials preparation. Nanogels are introduced as carriers for drug delivery and can be managed to allow oral delivery and brain bioavailability of low-molecular-weight drugs and biomacromolecules (Kabanov and Vinogradov 2009). An efficient nanogel carrier with broad biomedical capabilities should obtain acceptable stability in biological fluids, to prevent aggregation (Gonçalves et al. 2012). Different nanogel properties can be realised by modifying the functional groups, cross-linking density, and surface-active and stimuli-responsive elements. Nanogels show excellent potential for systemic drug delivery that should have some common characteristics such as a small (10-200 nm) particle size, biodegradability, biocompatibility, high stability, lengthy half-life, high levels of drug loading and/or entrapment, and protection from the immune system (Yallapu et al. 2011). Drug release from nanogels’ networks depends on the hydrophobic and hydrogen interactions and also, the harmonisation of drug molecules with polymer chain networks. Preclinical studies recommend that nanogels can be used for biopharmaceuticals delivery in cells in an efficient way (Maya et al. 2013). They also recommend that the nanogels can be used for increasing delivery of a drug across cellular barriers. Overall, the architecture of nanogel-bioactive conjugates can increase drug bioavailability, stability, loading efficiency, dispersibility, effective transdermal penetration, efficient targeting, and treatment ability against drug-resistant cancer cells and multicellular spheroids (Maya et al. 2013).

1.2.10. Polymeric-based Particles

Polymeric based particles are more common in drug delivery compared to lipid-based particles due to their tissue and organ targeting properties. They can also be classified as
synthetic, or natural polymers. The following are some of the common natural types of polymer-based particles utilised in drug delivery:

1.2.10.1. Chitosan

Chitosan is a positively charged biopolymer in the form of a linear polysaccharide derived from deacetylation of chitin by using sodium hydroxide. Chitosan is insoluble in water and organic solvents however, it is soluble in aqueous acidic solutions (pH < 6.5) and at lower pH values it forms a gel. Chitosan is commercially available in the form of powder, dry flakes or solution and has no toxicity in humans. Other properties, including biodegradability and good compatibility make it a unique substance to be used in the biomedical and pharmaceutical industries. One of the pharmaceutical applications of chitosan is to be applied as a carrier for specific drugs or components (Bernkop-Schnürch and Dünnhaupt 2012). Another important usage of chitosan is in wound healing as a result of some properties such as non-toxicity, anti-inflammatory effect, hemostatic action, biodegradability, biocompatibility, antimicrobial effect and stimulation of human dermal fibroblast activities. Figure 4 shows the structure of chitosan achieved by modification of chitin.
Figure 4. Structure of chitosan. Adapted from (Albuquerque et al. 2016) under creative common rules

1.2.10.2. Alginate

Alginate is a linear anionic biopolymer consisting of 1, 4-linked β-mannuronic and α-guluronic acid residues, which are obtained from algae. Alginate is extensively used in delivery systems because of its simple structure, biodegradability, and biocompatibility. Alginate is able to absorb water and make three-dimensional gels in the presence of calcium in aqueous solutions. Because of the mentioned properties, alginate can be used as a component of wound dressings as it can absorb body fluids up to 20 times its weight. The resulting hydrophilic gel provides a moist environment for the wound and helps the healing process. Figure 5 shows the structure of alginate.
The following are a few studies that have been conducted on the application of several drug delivery materials, and structures for overcoming the limitation of curcumin pharmacological action.

In a recent study, Das et al. (2010), designed a nanoformulation based on alginate (ALG) and chitosan (CS), two biocompatible biopolymers to deliver curcumin to cancer cells. They conducted initial studies by delivering the ALG-CS nanoparticles to HeLa cell lines. However, the results were not promising because of the hydrophilic nature of the polymers that caused low solubility of nanoparticles. In order to overcome the problem, they added pluronic F127 (PF127) and made a new composite. According to scanning electron microscopic and atomic force analysis, the particles had a spherical shape (100 ± 20 nm). Additionally, there was an increased efficiency in composite nanoparticles over the nanoparticles without PF127. Also, according to the cytotoxicity assay at 500 µg/ml concentration, the nanoparticles were non-toxic to the Hela cells. Furthermore, as curcumin is fluorescent it could easily be found in the cells, and the fluorescence microscope images proved internalisation of the curcumin loaded nanoparticles into the cells. Therefore, ALG-CS-PF127 composite nanoparticles of a suitable size distribution, encapsulation efficiency,
and release kinetics had the potential to deliver hydrophobic drugs for treatment (Das et al. 2010).

In another study, Song et al. (2011), developed a new formulation for curcumin delivery by applying a biodegradable and biocompatible poly (L-lactic acid) based poly (anhydride-ester)-b-poly (ethylene glycol) (PAE-b-PEG) micelle. They actually had mixed a polyester-based polymer with a polyanhydrides polymer in order to overcome the release problem of the nanopolymers. Polyester based polymers generally undergo bulk erosion and therefore degrade slowly, while polyanhydrides undergo surface erosion resulting in rapid degradation. Therefore, in order to develop a formulation with proper and controlled release kinetics, Song et al. (2011) applied a combination of both. The developed micelles were absolutely water-dispersible, overcoming the bioavailability problem. *In vitro* experiments indicated that curcumin micelles were taken up by endocytosis and showed increased cytotoxic activity on cancer cells compared to free curcumin. Additionally, the micelles exhibited stronger antitumor activity, increased anti-angiogenesis effects and apoptosis on the EMT6 breast tumour model. Also, *in vivo* safety tests including haematology analysis showed no sub-acute toxicity to major organs or haematological system in mice intravenous administration (Song et al. 2011).

Sahu et al. (2010) investigated the potential of a nanoformulation based on the two common Pluronic triblock copolymer micelles including Pluronic F127 and F68 for curcumin encapsulation. The potential of the micelles was examined by the encapsulation efficiency, *in vitro* cytotoxicity and *in vitro* drug release of the encapsulated samples. The encapsulated formulations were freeze-dried and reconstituted without using cryoprotectants. PF127 exhibited better efficiency in encapsulation and acceptable stability when stored for longer
periods compared to PF68. Physical interaction between curcumin and Pluronic was examined by XRD analysis, fluorescence, UV-visible and FT-IR spectroscopy. According to the AFM studies, the micelles had a spherical shape, and the diameters were below 100 nm. In addition, the *in vitro* cytotoxicity of the drug formulations was carried out via HeLa cancer cells. Also, encapsulated curcumin showed significant anti-cancer activity compared to free curcumin. Therefore, a formulation based on Pluronic is a favourable means of curcumin-delivery for clinical applications (Sahu et al. 2010).

Mukerjee et al. (2009) developed a carrier made of PLGA using solid/oil/water emulsion by a solvent evaporation method for encapsulating curcumin. The experiments included physicochemical characterisation plus cell viability and cellular uptake of the nanoparticles in prostate cancer therapy. Physicochemical experiments revealed that the nanoparticles had a spherical smooth surface with an encapsulation efficiency of around 90%. The size of the nanoparticles ranged from 35 to 100 nm, with the mean size of 45 nm. Studies using a fluorescence microscope showed an intracellular uptake of the nanoparticles in which the cells were exposed to 100 µg/ml concentrations of curcumin-loaded nanoparticles for up to 3 hours. Before that, the cells were labelled with Nile red and DAPI. The results of their investigation showed robust uptake of nanoparticles in all 3 prostate cell lines (Mukerjee and Vishwanatha 2009).

1.3. **Wound classification**

Wounds are generally classified as acute or chronic based on the time the wounds require healing. An acute wound can be described as a wound which heals within a short time and can be repaired on its own in a well processed, orderly and time organised procedure. Sustained physiologic and anatomical restoration of the function and integrity of the damaged
skin can thus be achieved (Nicks et al. 2010). Acute wounds generally occur as a result of traumatic injuries such as burns, abrasions and lacerations (Nicks et al. 2010). A chronic wound does not heal in a predictable, timely and well-programmed manner and fails to establish an absolute restoration of the skin damaged structure and function (Lazarus et al. 1994, Sen et al. 2009). Wounds that cannot heal in three months are generally regarded as chronic wounds and include pressure ulcers, venous ulcers, ischemic ulcers and diabetic foot ulcers (Sen et al. 2009, Posnett et al. 2009).

Pressure ulcers often happen in patients with insufficient mobility and extend the damage from skin to other parts of the body such as muscles, bones and tendons. In progressed levels, they can lead to infection, osteomyelitis, septicaemia, and in more advanced levels, they may even lead to death (Posnett et al. 2009, Sen et al. 2009).

Venous ulcers are usually produced as a result of venous hypertension (Billiar et al. 2004). Ischemic wounds, which are characterised by a pale base, are caused by a declined blood supply to the damaged parts that can lead to necrosis (Billiar et al. 2004).

Diabetic foot ulcers are one of the most common and prevalent complications that might take place in diabetic patients, and early diagnosis is a pivotal factor in avoiding these complications (Sen et al. 2009).

1.4. Natural wound healing mechanism

Skin serves as a natural barrier between an organism and its environment, protecting the organism from a variety of damages including physical, chemical and microbial. When the integrity of the skin is compromised by any injury, the body starts a continuous, multi-step complex process at the injured site as a response in order to restore the function of injured
tissues. The main principles of optimal wound healing are to minimise tissue injury and provide sufficient tissue perfusion and a moist environment to restore the function of the damaged site (Eming et al. 2007).

The above-described natural process of wound healing consists of four distinct but overlapping phases including homoeostasis, inflammation, proliferation and remodelling. Homoeostasis starts upon injury during which platelet aggregation and blood clot formation occur, which provides an extracellular cell matrix (ECM) for cell migration. Hemostasis is the most immediate response to the injury and happens as a result of damage to the blood vessels. Therefore, it is imperative to stop haemorrhage in damaged sites. This is achieved by activation and aggregation of platelets through damaged blood vessels, which leads to the formation of a clot composed of a network of insoluble fibrin fibres. The produced clot provides a bed for growth factors and migration of various cells (At 2008). The activated platelets are a major source of growth factors such as fibroblast growth factor, platelet-derived growth factor, vascular endothelial growth factor and transforming growth factor-β (TGFβ). These boost the different perspectives of the healing process and play an important role in inflammation, angiogenesis, migration of fibroblasts and keratinocytes (Bahou and Gnatenko 2004). Furthermore, serum, the fluid portion of clotted blood, is another effective factor in the repair process as it contains factors such as interleukins, tumour necrosis factor-α, colony stimulating factors, interferon-γ, and a series of other components that together lead to a serum response factor (SRF). SRF induces transcription of corresponding genes and various reports have indicated that up or down regulation of a broad spectrum of genes occurs as an early response to injury even during the first hour in a
The inflammatory response, as the second phase consists of the migration of the blood cells, including macrophages and phagocytic neutrophils towards the wound site. At the outset, the phagocytes remove foreign particles and towards the end of the phase they start to produce cytokines, which enhance the migration of fibroblasts.

The inflammatory response is initiated upon the leakage of neutrophils through injured blood vessels into the wounded area within minutes of injury (Kim et al. 2008). Neutrophils are responsible for cleaning the wound from invading microorganisms via various strategies such as recruitment of reactive oxygen species (ROS) (Dovi et al. 2004). Moreover, the induced expression of some genes in neutrophils in damaged parts is an obvious indicator of neutrophils effect in other aspects of wound repair such as the resolution of the formed clots, the progression of angiogenesis and also re-epithelialisation (Theilgaard-Mönch et al. 2004). Monocytes are dragged from the blood circulation later than neutrophils and transform into matured macrophages upon their entrance to the wounded tissue where they play a pivotal role in clearing up the matrix and cellular debris (Mori et al. 2008, Martinez et al. 2006).

Furthermore, the immune cells, which reside within the damaged site such as mast cells, T cells and Langerhans cells, are also stimulated, which in turn start releasing chemokines and cytokines (Noli and Miolo 2001, Jameson et al. 2004, Cumberbatch et al. 2000). Also, inflammatory cells can influence the surrounding tissue by ROS and NO formation. Although it is well known that NO and ROS drive definite aspects of tissue repair, the injured sites should perform some detoxifying strategies as a response to these stressors (Schäfer and Werner 2008, Sen and Roy 2008).
Therefore, the inflammatory response is started with the recruitment of neutrophils and macrophages from surrounded vessels, and is continued by induction of growth factor signals from the serum and inflammatory cells (Eming et al. 2007). These signals subsequently trigger expression of selectins, which control the passage of leukocytes to the vessel wall, and crossing of the endothelial hedge (T. J. Shaw and Martin 2009).

A large number of studies have been performed to compare the gene signatures of different wounds with and without inflammation. Although there are still debates about whether the presence of inflammatory cells is a vital requirement for tissue repair or not, studies have suggested that these cells can affect all the other cells at the injured site, as well as in the surrounding tissue (Martin and Leibovich 2005). For example, it has been well established that one of the main functions that inflammatory cytokines exhibit together with signals from other cells during the repair process is angiogenesis (Tonnesen et al. 2000).

The third phase of wound healing is characterised by the formation of new blood vessels (angiogenesis or neovascularisation), which helps to sustain the new tissues and the fabrication and deposition of ECM protein such as collagen and granulation tissue and epithelialisation. Overall proliferation, migration and contraction, which involve the actions required to a temporary closure of wounds and renewal of the lost tissue happen in this phase. This process starts within hours of the injury and will terminate at different times depending on the size and location of the damaged site, as well as the health condition and age of the patient. Angiogenesis, which is imperative to a successful wound healing involves the formation of blood vessels and initiates with an endothelial cell bud migrating through the wound gap (Fisher et al. 1994). The endothelial cells then divide and develop tubular structures, which connect with other buds and differentiate into capillaries, venules and
arterioles accordingly (Martin 1997). The capillaries start to form a microvascular network, which will be evident within a few days of injury. The microvascular network supplies oxygen and nutrients to the damaged tissues and assists in developing the provisional matrix called granulation tissue (Tonnesen et al. 2000).

The dominant acting cells in this phase are keratinocytes and fibroblasts, which reside in the epidermis and dermis respectively. Re-epithelialisation of the wound, which proceeds in parallel to angiogenesis is achieved through migration together with the proliferation of keratinocytes in the wound gap. Generally, keratinocytes migrate across the wound collectively after experiencing a series of modifications to ease their movements. Keratinocytes modify cell-cell and cell-matrix bonds to potentiate their development from a collagen-IV-rich BM and laminin-V onto the provisional matrix layers (T. J. Shaw and Martin 2009). Likewise, they induce the expression of proteinases such as matrix metalloproteinases (MMPs) (Pilcher et al. 1999). Fibroblasts are the dominant cells in the dermis that generate the new extracellular matrix called granulation tissue using collagen fibres as building units, which are crucial for tissue consistency (Garlick and Taichman 1994). Fibroblasts are drawn from different sources including the healthy and intact dermis at the wound site, circulating fibrocytes, bone marrow pioneer cells and the pericytes, which are associated with adjacent blood vessels (Hinz 2007, Abe et al. 2001, T. J. Shaw and Martin 2009). Collagen is a protein essential to the wound healing process and is known as the most crucial connective tissue protein. There are several types of collagens according to the structures they form among which type I and III are the most common identified in wound healing. Generally, collagen fibres are formed in a basketweave design in healthy tissues. However, this systematised structure cannot be formed at the wound site as fibroblasts nearby
the wound area align collagen fibres in concert to the stress lines of damaged tissue (Singh et al. 2013). The formed stress fibres, consisting of actin bundles, which are able to contract weakly, enable connective tissue contraction and the wound starts to shrink by recruiting nearby tissue and drawing it into the wound. Fibroblasts are modified in a way that not only allows them to move across the wound and be mobile, but also simultaneously, can pull the edges of the wound to the centre of the wound (Singh et al. 2013). In the extracellular matrix, collagen helps in maintaining the cells in place and enhances the contraction process, and re-epithelialisation happens as a part of this phase (Gilbertson et al. 2001). Hence, fibroblasts, and more specifically myofibroblasts, play a crucial role in this phase by contributing to the formation, bundling and arrangement of collagen fibres, the principal component of scar tissue (Singh et al. 2013).

The last phase of wound repair, which is characterised by tissue remodelling, epithelialisation and scar formation is essential for full restoration of the function and appearance of the damaged site. Several changes occur, during this phase at both epidermis and dermis level. Keratinocytes, which act through migration and proliferation in the previous phase, confront one another at the wound margins as the wound closes, after which they stop acting (T. J. Shaw and Martin 2009). The blood vessels of the scar get refined and modified to generate a functional network. ECM, which was formed in the early stages is remodelled, which restores the normal structure of the dermis. Later in this phase activation of some enzymes such as MMPs occurs which results in degradation of ECM proteins at the injured site (T. J. Shaw and Martin 2009). Endothelial cells and myofibroblasts undergo apoptosis and macrophages are deactivated by cytokines or phagocytosis. Overall, the quantity of collagen declines whereas tensile strength increases due to the physical alterations of the newly formed
collagen (Enoch et al. 2006). Figure 6 indicates the different phases of wound healing process.

**Figure 6.** Schematic process of wound healing process

1.5. **Diabetes**

Diabetes mellitus is a chronic disease and metabolic disorder described by high blood glucose levels. Based on the statistics, in 2010, 285 million people had diabetes and Shaw et al. (2010) reported that the number would increase to 439 million by 2030, which represents a 54% increase (J. E. Shaw et al. 2010). A variety of factors will contribute to this substantial
increase including decreased physical activity and exercise, obesity, ageing of the populations and modernisation of lifestyle. The above-mentioned figure for the incidence of diabetes is assessed to have a 42.4% rise in North America, which was the most dominant part of the world for diabetes incidence in 2010, and 65.1% rise in South, and Central America by 2030. Europe, Pacific, and South Asia are expected to experience a 20, 47, and 72% increment in diabetes prevalence, while Africa is predicted to have a drastic increase of 98% in adults by 2030. According to the study by Shaw et al. (2010) the diabetic population will be evidently higher in developing countries by 2030 as the average population age will slightly rise (J. E. Shaw et al. 2010)

Figure 7, compares diabetes outbreaks in 2010 and 2013, in different parts of the world.

If diabetes is not properly controlled, it might lead to various problems such as obesity, stroke, coronary heart disease, diabetic retinopathy and diabetic nephropathy. Also, diabetes contributes to numerous complications of the foot including foot ulcer, osteomyelitis and Charcot’s neuroarthropathy. According to the literature, Australia’s national health expenditure on chronic wounds such as foot ulcers is estimated to be approximately 500 million dollars per year (McGuiness and Rice 2009). The chronicity of wounds has become a major problem not only because of the economic burden of the clinical costs, but also its impact on patients’ emotional well-being and quality of life (McGuiness and Rice 2009). Hence, it is imperative to conduct further investigations in the field of chronic wounds and their management in order to improve both economic and social aspects of patients’ lives.
Figure 7. Prevalence of diabetes in the world in 2010 and 2030. Adapted from (J. E. Shaw et al. 2010)

1.6. Diabetic foot ulcer

Whilst the sequence of healing occurs orderly and in a well-orchestrated pattern in acute wounds eventually leading to wound closure, in diabetic wounds this process is disrupted and results in non-healing wounds that are arrested in one, or more of the aforementioned phases (Falanga 2005). Diabetic neuropathy known as nerve tissue damage, which is a common complication occurring in diabetic patients, is characterised by an enhanced loss of peripheral nerve fibres as a result of diminished blood flow and increased glycemic levels (Dyck et al. 1993, Bašić-Kes et al. 2011). Diabetic neuropathy can occur in both diabetes type I as well as type II, and is more frequent in aged patients. Typically, the symptoms of neuropathy emerge 10-20 years following diagnosis of diabetes and almost 50% of the diabetic cases develop nerve damage to some extent (Rathur and Boulton 2005). While many patients are prone to developing this complication even at early stages, others might never experience it (Boulton et al. 2004). According to a wide range of studies, numerous factors
contribute to the emergence of foot ulcers. Vascular diseases and ischemia disrupt the healing process because of the decreased levels of oxygen and nutrients conveyed to the damaged tissue (Guo and DiPietro 2010). Also, enhanced nitric oxide levels, which lead to increased formation of ROS, along with downregulation of glutathione and cysteine contribute to impaired healing (Brem and Tomic-Canic 2007). Additionally, abnormal extension of the inflammatory phase originating from impaired expression of growth factors such as TGF-β contributes to this pathology (Tsunawaki et al. 1988). There are reports indicating decreased production of cytokines and macrophage dysfunction, consequently leading to an impaired capacity of macrophages to clear the wound site from necrotic materials, which is essential for a proper and organised healing process (Khanna et al. 2010, Mirza and Koh 2011, Robert Blakytny and Jude 2009, Liu et al. 1999).

The factors which contribute to impaired healing in diabetes during the angiogenesis phase include downregulation of hypoxia inducible factor-1α (HIF-1α), which is responsible for transcription of some growth factors such as vascular endothelial growth factor (VEGF), thereby the levels of VEGF will be diminished (Catrina et al. 2004, Botusan et al. 2008). A study by Pradhan et al. (2011) revealed decreased expression of several other angiogenesis promoting growth factors, such as insulin like growth factor 1 (IGF-1), epidermal growth factor (EGF), platelet derived growth factor (PDGF) and interleukin-8 (IL-8), which stimulate cell proliferation, and migration (Pradhan et al. 2011). A synchronised formation of ECM is of great importance in the process of wound healing, as different endothelial cells migrate in this way and use ECM as a scaffold while creating new blood vessels during neovascularization. Hence, finally and most significantly, excessive activity of matrix metalloproteinases (MMPs), or downregulation of MMP inhibitors, represent the most
destructive factors in diabetic wound healing process. MMPs can disrupt the healing procedure by breaking down growth factors, as well as ECM components such as collagen, leading to poorly developed ECM and consequently impaired re-epithelialisation and impaired wound healing (Natraj et al. 2015, Stricklin et al. 1993, Han et al. 2001). Figure 8 illustrates the healing process in regular wound healing compared to the diabetic wound. The clinical treatment of skin injury due to different severe burns or wounds continues to be a significant problem. A prominent therapeutic agent should be able to augment one or more phases of the wound healing process without making detrimental side effects.
Figure 8. Wound healing process in normal and diabetic wounds. Reproduced with permission from (Moura et al. 2013)
1.7. Effects of curcumin on different phases of wound healing and mechanism of action

According to the previous published studies, curcumin can influence different stages of wound healing including inflammation, proliferation and contraction.

1.7.1. Effect of curcumin on inflammation

Inflammation is the second most serious stage in wound healing and is often known as the first decisive step in optimum recovery. Having control over immediate acute inflammation of skin injury is essential. Indeed, uncontrolled inflammatory reactions can lead to serious and sometimes severe subsequent tissue damages which can be seen in rheumatoid arthritis as an inflammatory disorder (Singer and Clark 1999). Joe et al. (2004) studied the mechanism of action of curcumin on inflammation and summarised:

- Ability to inhibit the production of two main cytokines released from macrophages and monocytes that have a major impact on the regulation of inflammatory responses comprising tumour necrosis factor alpha (TNF-α), and (IL-1).

- Ability to inhibit activity of NF-(κ) B (nuclear factor kappa-light-chain-enhancer of activated B cells), which has been considered as an oxidant-responsive transcription factor that regulates many genes involved in the initiation of inflammatory reactions (Frey and Malik 2004).

- Reducing oxidation (a major cause of inflammation) by exhibiting scavenging action on reactive oxygen species (ROS) (Joe et al. 2004).
Curcumin has been shown to reduce inflammation which consequently leads to the acceleration of the transition to the subsequent phases including proliferative and remodelling.

1.7.2. Effect of curcumin on the proliferative phase of wound healing

This phase includes four stages of fibroblast proliferation, granulation tissue formation, collagen deposition and, apoptosis. The effect of curcumin on all these stages has been investigated as below:

1.7.2.1. Effects of curcumin on the fibroblast proliferation

The presence of fibroblasts in the wound and damaged sites is of essential importance for the damaged tissue to be healed. According to the study by Blakytny and Jude (2006), cutaneous wounds, which fail to be healed in an appropriate time period usually have impaired fibroblast proliferation and migration in the damaged area. The infiltration of fibroblasts into the damaged site is of significant importance for granulation tissue formation/remodelling, collagen production and remodelling (R Blakytny and Jude 2006).

Several studies have shown the positive effect of curcumin on the infiltration of fibroblasts into the wound area. Mohanty et al. (2012) investigated the formulation of a curcumin loaded polymeric bandage in a rat model. The biochemical analysis revealed enhanced and faster wound reduction and increased cell proliferation of the samples. The accelerated effect of the bandage was due to early implementation of fibroblasts and their differentiation (Mohanty et al. 2012). However, there are limitations in the application of curcumin. In contrast Scharstuhl et al. (2009), revealed that higher concentrations of curcumin (25 µM) caused cell
apoptosis turning oxidative, but at lower concentrations positive results were achieved without any indication of cell death (Scharstuhl et al. 2009).

1.7.2.2. Effects of curcumin on the stage of granulation tissue formation

Almost four days after skin injury, new stroma or granulation tissue starts to grow. This process is characterised by the development of new small capillaries and the infiltration of fibroblasts, which helps the formation of extracellular matrix (Singer and Clark 1999). The newly formed tissue helps the migration of the epithelial cells and wound closure and improves re-epithelialization. Mohanty et al. (2012), demonstrated that the wounds in rats, which had been treated by COP (curcumin-loaded acid, oleic polymer) showed a better alignment of granulation tissue 10 days post-treatment and just a marginal improvement four days after injury (Mohanty et al. 2012).

1.7.2.3. Effect of curcumin on the stage of collagen deposition

Another main pre-requisite for the wounded site to be healed in a suitable period is the remodelling of the extracellular matrix, which is composed of different proteins such as collagen that mainly contributes to 70-80 % of the skin. The last purpose in the wound healing process is the establishment of scar tissue, which mainly consists of collagenous fibres. Therefore, the formation of adequate collagen and its deposition contributes to wound repair to a large extent. Dai et al. (2009) demonstrated that the content of collagen increased in rat models treated with curcumin sponge, and that they were thicker and had been organised in more compact rows compared to controls (Dai et al. 2009). Similarly, another study by Panchatcharam et al. (2006) showed not only increased amount of collagen in topically
curcumin treated wounds in rats, but also that collagens were able to mature faster. Another result of their study was the increase in the amount of aldehyde in collagens, which is an indicator of the high cross-linked structure of the produced fibres (Panchatcharam et al. 2006, Mohanty et al. 2012). This finding also confirmed by Mohanty et al. (2012), demonstrated an increase in collagen level as well as a higher content of aldehyde in a rat model wound, treated with curcumin bandage compared to the control.

1.7.2.4. **Effect of curcumin on the stage of apoptosis**

During the wound healing process, apoptosis of a group of unwanted inflammatory cells is as essential as the growth of some other cells, in order to pass the inflammatory stage and achieve the next stage. Scharstuhl et al. (2009) showed that curcumin could cause apoptosis due to its ability to induce ROS, even though there was little information on its mechanism of action (Scharstuhl et al. 2009). Mohanty et al. (2012), using a rat model, reported the apoptotic effect of curcumin at the four days post-treatment stage when treated with curcumin bandages. They demonstrated that the rate of apoptosis was low at the early stage of wound healing in control samples whereas in curcumin treated samples the transition from the early stage to the proliferative stage had been accelerated (Mohanty et al. 2012).

1.7.3. **Effect of curcumin on the stage of wound contraction**

Wound contraction, which is the part of the ultimate stage of wound healing, occurs when fibroblasts switch to myofibroblasts around two weeks after the injury. This stage is a complex stage consisting of different interactions between extracellular matrix proteins, cells and cytokines. Durgaprasad et al. (2011) reported that curcumin was able to augment wound
contraction, which leads to wound healing acceleration. By measuring the size of a wound and tracking it during the post-wounding period, they showed that curcumin-treated samples had a 20% increase in the wound contraction rate (Durgaprasad et al. 2011). Dai et al. (2009) showed a 16% increase in wound contraction following curcumin sponge treatment compared to controls (Dai et al. 2009).

1.8. Research gap
A review of the literature shows that there is extensive evidence that curcumin possesses a number of therapeutic effects, most notably in wound healing. However, delivery and bioavailability issues, arising from poor solubility, rapid metabolism in the body and chemical instability, limited the practical development of curcumin as a promising drug candidate. Curcumin is more suited as a wound healing agent for topical applications rather than for oral administration. A number of delivery vehicles have been formulated with curcumin in order to enhance its wound healing effects. These included collagen films (Gopinath et al. 2004), chitosan-alginate sponges (Dai et al. 2009), alginate foams (Hegge et al. 2011), polymeric bandages (Mohanty et al. 2012) and creams (Durgaprasad et al. 2011). These formulations have been shown to increase the wound healing effect of curcumin compared to the application of free curcumin itself. However, there is rather limited research on the potential of curcumin delivered in the encapsulated form as a topical agent for wound healing with the possibility of delivering better outcomes than the other formulations. Optimisation of physicochemical characterisation of encapsulated curcumin as a wound healing agent are yet to be established.
This thesis addresses this research gap by attempting to develop a biodegradable and biocompatible polymeric composite for curcumin encapsulation, to establish the optimum formulation conditions, to evaluate the drug release characteristics and to apply the encapsulated curcumin in a wound healing cell line model.

1.9. Research hypotheses

The hypotheses addressed in this thesis were that:

1- Encapsulation of curcumin in a polymeric-based composite consisting of alginate, chitosan and Pluronic F127 will improve solubility and stability compared to free curcumin.

2- Pluronic F127 increases the encapsulation efficiency and release of curcumin with alginate and chitosan.

3- Curcumin-loaded particles are taken up by human keratinocytes cells and display less inhibitory effects on cell growth compared to free curcumin.

1.10. Research aims

In order to test the hypotheses, the aims of the thesis were to:

1- Establish optimal formulation parameters for the encapsulation of curcumin with alginate and chitosan, with or without Pluronic F127.

2- Determine the physicochemical characteristics of the curcumin particles, which would include

   ▪ Examination of particle shape by Transmission Electron Microscopy (TEM)
- Determination of zeta potential, polydispersity and size of the curcumin-loaded particles
- Determination of curcumin encapsulation efficiency
- Studying \textit{in vitro} release kinetics of curcumin from particles

3- Determine the effects of curcumin loaded particles on cell viability, cell cytotoxicity and cellular uptake using a human keratinocyte cell line.
Chapter 2: Materials and methods

2.1. Materials
Sodium alginate (low viscosity), chitosan (low molecular weight, with 75-85% deacetylation degree), Pluronic F127 (PF127), calcium chloride (CaCl₂) and curcumin (≥ 94% curcuminoid content, ≥ 80% curcumin) were all purchased from Sigma Aldrich. All the chemicals were of analytical grade. MilliQ water was provided by the laboratory.

2.2. Equipment
UV-VIS spectrophotometer (LKB Navaspec II, model 80-2088-64, serial no: 53652), sonicator (Ultrasonic cleaner, Unisonics, type: FxF8M), centrifuge (Eppendorf, model: Centrifuge 5415C), ultracentrifuge (Beckman Coulter, model Avanti JE, 875892) were the major appliances used for this project. All the equipment were provided by Chemistry and Microbiology Laboratory, Faculty of Veterinary and Agricultural Sciences (FVAS), The University of Melbourne.

2.3. Preparation of blank alginate/chitosan/PF 127 particles

2.3.1. Preparation of stock solutions
Sodium alginate and calcium chloride solutions (0.63 and 2 mg/ml) were prepared by dissolving 14.36 and 2.72 mg respectively in milliQ water. Chitosan solution (0.47 mg/ml) was prepared by dissolving 2.24 mg in 1% (vol/vol) acetic acid. Curcumin solutions were
prepared in ethanol at a concentration of 1 and 2 mg/ml and pH values of alginate and chitosan solution were adjusted to 5.2 and 4.9 respectively.

2.3.2. Preparation of the blank particles

Particles were prepared based on the ionotropic gelation at room temperature according to Rajaonarivony et al. (1993), with some modifications. Briefly, PF127 was added at the required concentrations (0, 0.1 and 1% w/v) to 1.36 ml calcium chloride (2 mg/ml), and the solution was added to 22.8 ml sodium alginate solution (0.63 mg/ml) under mild magnetic stirring for around 30 min, in a dropwise manner. After 15 min, 4.78 ml of chitosan solution (0.47 mg/ml) was added in the same manner and stirred for 45 min. The resultant solution was equilibrated overnight at room temperature to allow the particles to form (Das et al. 2010, Rajaonarivony et al. 1993).

2.3.3. Preparation of curcumin loaded particles

CaCl$_2$ solution (2 mg/ml) was added to curcumin solutions (1 and 2 mg/ml). After that PF127 solution, was added at the required concentrations (0, 0.1 and 1% w/v) to the resultant curcumin-CaCl$_2$ solution, and the next steps were followed as explained above for blank particles.

Figure 9 schematises the particle formation process, and the ultracentrifuge utilised in fabrication procedure.
Figure 9. Schematic process of curcumin particle fabrication
2.4. Optimisation of the procedure parameters

The characteristics of the fabricated particles were investigated by applying various concentrations of curcumin solution (1 and 2 mg/ml), PF127 as a non-ionic surfactant (0, 0.1 and 1% w/v), and rinsing methanol/water solution (20/80, and 50/50). The optimum formulation was chosen on the basis of the total physico-chemical characteristics including, particle size, charge, encapsulation efficiency, and release studies.

2.5. Physicochemical characterisation of the particles

2.5.1. Particle size, zeta potential and polydispersity index

Characterisation of hydrodynamic size and zeta potential and PDI was performed by a Zetasizer Nano-ZS Malvern. Particles size indicates the diameter of the particle and PDI is an indicator of particle size distribution and stability. Zeta potential provides information about the difference between the potential of the dispersion fluid and the attached layer of the fluid to the particles.

All the measurements were performed using the 1:10 (v/v) diluted suspension of particles in milliQ water with an equilibrium time of 120 second, and 20 runs. During the whole process the temperature was kept at 25°C and 3 replicates were done for all 3 parameters.

2.5.2. Morphology determination by transmission electron microscopy (TEM)

In order to obtain precise morphological characterisation of the particles, transmission electron microscopy (TEM) was applied to 4 sets of particles including alginate-chitosan-PF127-curcumin at 0.1% concentration of PF127, and 0.1% curcumin, as the drug-loaded particles and also the control particles without curcumin in the presence of 0%, and 0.1%
PF127. The particles were suspended in distilled water and a drop of each sample was placed on a carbon-coated copper grid, which was left for a few minutes to be air dried at room temperature. Then the samples were loaded into the TEM microscope model Philips CM120 BioTwin.

2.5.3. Encapsulation efficiency of loaded curcumin

To determine the encapsulation efficiency of the fabricated particles, the particle solution was centrifuged at 20,000 rpm at 4°C for 20 min. The supernatant was removed, and the pellet was washed twice with a mixture of methanol and distilled water based on the experimental design, in order to remove any unbound curcumin. The curcumin in both supernatant and the rinse washes were analysed and quantified spectrophotometrically at 425 nm, which corresponded to the maximum absorbance of curcumin.

Therefore, the amount of non-encapsulated curcumin (free curcumin) was calculated based on a calibration curve equation:

\[ y = 1586x - 0.1614 \quad (R^2 = 0.978) \]

\( y \) is the absorbance, and \( x \) is the amount of curcumin. Encapsulation efficiency was calculated as follow:

Encapsulation efficiency (%) = (Total of curcumin - Total Free curcumin) / Total of curcumin

Total free curcumin is the amount of free curcumin in the first supernatant plus the amount of free curcumin in rinse supernatants.

Figure 10 shows the standard curve of curcumin in methanol applied in determination of encapsulation efficiency.
2.5.4. *In-vitro* curcumin release studies

To perform the release study of encapsulated curcumin, dialysis methodology was applied. Dialysis tubes were soaked in distilled water and then rinsed with phosphate buffered saline (PBS) to remove preservatives. Then 2 ml of curcumin loaded particle suspension was placed in the dialysis tube and sealed. For each experiment 8 tubes were prepared and dispersed in 600 ml PBS solution at pH 7.4. The solutions were placed in a shaking incubator under stirring at 120 rpm at 37°C. At determined time intervals, one of the samples was withdrawn and evaluated spectrophotometrically at 425 nm based on the standard curve of curcumin explained above. The release was calculated as below:

Release (%) = Released curcumin / Total curcumin ×100

Figure 11 shows different solutions of curcumin, and the dialysis tube used in release kinetic experiment.

![Standard curve of curcumin in methanol](image)

**Figure 10.** Standard curve of curcumin in methanol
2.6. Cell culture

The human FEP-188 keratinocyte cell line transformed by human papillomavirus, were derived from neonatal foreskin (provided from Peter McCallum Cancer Institute, East Melbourne), and cultured in serum-free growth medium (K-SFM) (Life Technologies), containing L-glutamine, EGF and BPE, supplemented with 2.5 µg EGF human recombinant, and 25 mg bovine pituitary extract. The cells were maintained in an incubator at 37°C in a humidified atmosphere containing 5% CO₂ up to the point when they reached 80% of confluence.

2.7. Routine cell culture

Human FEP-188 keratinocyte cell line, which was cryopreserved using 10% DMSO, was initially thawed and added to 10 ml of K-SFM media, then centrifuged at 1,200 rpm for 5
min, at room temperature using an Allegra X-22R centrifuge (Beckman Coulter, California, USA). Following the removal of the supernatant, the cells were suspended in 10 ml of the respective media, then transferred to a flask and incubated within a humidified atmosphere including 5% CO$_2$ at 37°C (Sanyo, Osaka, Japan). The cells were grown as a monolayer within the flask and were visualised on a daily basis using an Olympus CKX41 phase contrast microscope (Olympus, Tokyo, Japan) at 20X magnification. At appropriate time intervals, the media was changed to supply the cells with enough nutrients for continued growth.

### 2.8. Preparation of cell culture for treatments

Once the cells reached about 80% confluence as a monolayer within the flask, the medium was aspirated and washed two times with 10 ml of PBS. Subsequently, 5 ml of trypsin-ethylenediaminetetraacetic (trypsin-EDTA) 0.05% (v/v) (Life Technologies) was used to trypsinise the cells until the morphology of the cells switched from attached, elongated, dark cells to detached, round, bold and bright cells. Then immediately 7 ml of supplemented DMEM was used to inactivate the trypsin-EDTA, as K-SFM lacks FBS (fetal bovine serum), which is necessary to inactivate the trypsin. This step was followed by pipetting the suspension against the back wall and back corner of the flask, each for ten times, in order to get a single cell suspension, and subsequently transferred to a 15 ml falcon tube and centrifuged at 1,200 rpm, for 5 min at room temperature. The supernatant was discarded and the cell pellet was suspended in 10 ml of the corresponding media, reverse pipetted in order to achieve a uniform cell suspension, before being transferred to a T75 cm$^2$ flask containing medium.
In order to determine the number of cells required for the experiments, 10 µl of the above-mentioned suspension was removed and the cells were counted using a haemocytometer in 4 fields of view and the number of the cells were estimated based on the mean of the mentioned four fields.

2.9. Drug preparations for cell treatments

Free curcumin is not soluble in water, therefore first it was dissolved in DMSO and then diluted with sterile PBS. The highest concentration of DMSO did not exceed 0.4% for cell treatments. Encapsulated curcumin particles were directly suspended in sterile PBS and administered to the cells.

2.10. Cell viability assay

In order to determine the susceptibility of FEP-188 keratinocyte cells to free and encapsulated curcumin, a cell viability assay was performed using Cell Titre Blue® reagent (Promega, Winchester, USA). For economy of resources, the FEP-188 keratinocyte cell line was applied as a representative of skin cells in our study. FEP-188 keratinocyte cells were plated at a density of 3 × 10^4 cells per well in a 96 well flat bottom black sterile culture plate (Promega) and incubated to let the cells attach to the wells. After 24 h, the cells were treated with different concentrations of free curcumin dissolved in DMSO, and curcumin particles (0, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 15, 20, and 25 µg/ml). This range was selected based on the literature review (Scharstuhl et al. 2009, Phan et al. 2001) Untreated cells served as controls. Then the cells were incubated, for another 24 h. After cell treatment with the drug, 20 µl of Cell Titre Blue® reagent was added to each well, which was followed by incubation
for 4 h. The procedure was terminated after 5 days, and cell proliferation was assessed by fluorescence intensity at 550 nm excitation, and 615 nm for emission using the multilabel plate reader (Perkin Elmer, Massachusetts, USA); (Boonkaew et al. 2014, Böhmert et al. 2012). All the experiments were performed in triplicates.

IC50 or half maximal inhibitory concentration is a specific concentration of the drug which inhibits cell growth by 50%. Cell Titre Blue reagent is a buffered solution containing pure resazurin dye, which is used to evaluate the metabolic activity of cells, and consequently, indicates the cell viability. Viable cells retain the ability to reduce resazurin to resorufin, and produce fluorescent signals, while nonviable cells lose their capacity to reduce resorufin and thus cannot generate a fluorescent signal. Resazurin is dark blue in colour and produce low intrinsic fluorescence until it is reduced to resorufin, which is pink in colour and strongly fluorescent. Resazurin is reduced to resorufin as a result of various redox enzymes released from mitochondrial, microsomal, and cytosolic sites.

2.11. Microscopic cell toxicity

Morphology analysis was carried out to verify the microscopic features of the FEP-188 keratinocyte cells treated with different concentrations of curcumin particles and free curcumin dissolved in DMSO. FEP-188 keratinocyte cells were plated at a density of 2 × 10^5 cells per well, in 6-well culture plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂ to let the cells become confluent. After 24 h, the cells were treated with four different concentrations of free curcumin dissolved in DMSO and equivalent concentrations of encapsulated curcumin, followed by incubation of the cells for another day. Untreated cells served as controls. The procedure was terminated after 3 days.
2.12. **Cellular uptake by confocal microscopy imaging**

Having determined the physicochemical characteristics of different formulations, the ability of the optimal formulations to internalise FEP-188 keratinocyte cells was investigated by confocal microscopy. Curcumin is a fluorescent compound and can therefore be tracked inside the cell by examining fluorescence without the need to tag a fluorescent dye to the compound. Therefore, taking advantage of the intrinsic photochemical property of curcumin, an investigation on curcumin intracellular uptake by FEP-188 keratinocyte cells was conducted.

To prepare the samples for microscopic imaging, FEP-188 keratinocyte cells were cultured in serum-free growth medium (K-SFM) containing L-glutamine, EGF, BPE, supplemented with EGF human recombinant and bovine pituitary extract. Subsequently, cells were seeded at a density of $10^5$ cells on a coverslip in a 6-well tissue culture plate, followed by incubation at 37°C in a humidified atmosphere containing 5% CO$_2$. After 24 h the cells were exposed to 20 µg/ml of free and encapsulated curcumin, and confocal scanning microscopic imaging was carried out on the control cells, cells with free curcumin in PBS, cells with empty particles and cells with curcumin particles over a time-dependent period. Fluorescent images of the treated and untreated cells were monitored with a Nikon A1r confocal microscope (Nikon, Tokyo, Japan).

2.13. **Statistical analysis**

SPSS software (version 23) was applied for the statistical analysis. All the data were expressed as mean ± SD. One-way ANOVA was utilised for comparing the mean values, and
multiple comparisons were made using Tukey’s multiple comparison tests. Also, T-test was used whenever required. Statistical significance was determined at $P < 0.05$. 
Chapter 3: Results and discussion

3.1. Physicochemical experiments

3.1.1. Effects of methanol (%) rinse on particles

The concentration of methanol was optimised to enable separation of free from encapsulated curcumin while retaining particle integrity. Higher concentrations of methanol (%) rinse, resulted in significantly lower encapsulation efficiency at lower curcumin concentrations. However, higher curcumin concentrations resisted methanol wash and the difference was not significant (0.2%) (Figure 12). Also, the effect of different methanol (%) rinse concentrations was not significant on other parameters including particle size, PDI, and zeta potential (p value > 0.05) (The data are not shown).
3.1.2. Effects of PF127

Up to 1% (w/v) PF127 was used and the results from previous reports were corroborated. PF127, the non-ionic surfactant, decreased the surface tension and helped to increase the solubility of curcumin in the hydrophilic fluid. The increase in the concentration of PF127 from 0% to 1% (w/v) enhanced the encapsulation efficiency in all formulations (Figure 13). This could be attributed to the increased solubility of curcumin in the aqueous phase, which consequently led to a marked enhancement in the encapsulation efficiency of the particles. This finding is in accordance with the study by Rajath et al. (2013), which reported that incorporation of PF127 to the formulation at an appropriate concentration resulted in a higher
encapsulation efficiency. Also, Das et al. (2010), found that addition of PF127 to the formulation resulted in a 5-10 fold increase in the final encapsulation efficiency. Bhunchu et al. (2015), demonstrated that an increase in the PF127 concentration from 0% to 1% (w/v) resulted in an increase in the encapsulation loading capacity of the particles. However, further increase in PF127 concentration to 1.5% (w/v) decreased the resulting encapsulation efficiency.

A variety of surfactants are used in the pharmaceutical and cosmetic industries. Various surfactants have been used in previous studies to decrease the surface tension between hydrophobic and hydrophilic phases. The safety of non-ionic surfactants over anionic or cationic ones is well established and satisfactory results have previously been reported with PF127 (Raveendran et al. 2013, Sahu et al. 2010, Lippens et al. 2013).

The addition of 0.1% PF127 to the solution significantly decreased the particle size. However, the size was increased at above 0.1% PF127 (Figure 13). This can be attributed to the stabilisation effect of PF127, which inhibits the aggregation of the particles by steric stabilisation (Bhunchu et al. 2015). Hence the size was decreased in the presence of PF127. However, the increase in PF127 concentration beyond 0.1% raised the viscosity of the aqueous phase, which resulted in the formation of larger particles. These results were further supported by Bhunchu et al. (2015), who reported the increase in particle size at 1% PF127.
Figure 13. Variation in curcumin encapsulation efficiency, and particle size by variation of PF127 concentration and methanol rinse (MR) at a: 0.1% curcumin concentration, and b: 0.2% curcumin. All data represent mean ± SD (n = 3), and *p value < 0.05
PDI significantly increased at 1% PF127. However, no significant changes were seen at a range of concentrations between 0% and 0.1% of PF127 (Figure 14).

The zeta potential of the particles was negative as alginate formed the core part of the particles. The zeta potential became slightly less negative with a proportional increase in PF127 concentration % (Figure 14). These phenomena may have been due to the micelle forming behaviour of PF127 around curcumin, and the increased interaction of PF127 with the polymeric system, by its polar head groups. Hence, the increase in PF127 concentration from 0.1% to 1% can enhance the interaction of PF127 with the polymer network, neutralising the negativity of the particle surface and resulting in the formation of larger particles.

![Chart showing changes in PDI and zeta potential with PF127 concentration](image)
3.1.3. Effects of curcumin concentration on zeta potential

The zeta potential values were within the range of -6.60 to -17.50 mV for different curcumin loaded particles, and -14.4 to -23.5 mV for the blank particles which were relatively lower than the same polymeric matrix applied in previous studies conducted by Bhunchu et al. (2015), and Fattahpour et al. (2015). A significant difference in zeta potential occurred between the curcumin-loaded particles, and the blank particles without curcumin, indicating more stability of the blank particles compared to curcumin loaded particles.

Zeta potential is a parameter which provides information on the stability of the dispersions as well as information about the surface charge of the particles. Hence, zeta potential can specify the interaction level between the particles. Generally, zeta potential values above +30
mV or below – 30 mV, is considered stable (Bhunchu et al. 2015). The mentioned range for zeta potential is strong enough to maintain the repulsion between the particles, and to avoid the aggregation of the particles. Higher rates of increase or decrease in zeta potential intensifies the repulsion between particles ultimately resulting in the formation of more stable dispersions, along with more uniform and narrow size distribution. Wu et al. (2011), suggested that particle suspensions containing PF127 were stable within the zeta potential range of -20 to -30 mV (Wu et al. 2011). Figure 15 depicts the zeta potential graphs of blank particles, and optimised curcumin-alginate-chitosan-PF127 particles.
Figure 15. Zeta potential plot of curcumin particles a: empty particles (blank) 
b: formulation with 20% methanol rinse, c: formulation with 50% methanol rinse
3.1.4. Effects of curcumin concentration on particle size

The addition of curcumin to the system produced significantly larger particles compared to the blank particles without curcumin (P < 0.05) (Figure 16). However, no significant increase in size was seen while different concentrations of curcumin were applied. There were no significant changes in PDI, zeta potential and encapsulation efficiency of different formulations.

**Figure 16.** Variation in particle size by variation of PF127, and methanol rinse concentration at different concentrations of curcumin. All data represent mean ± SD (n =3), a, b p value < 0.05 between different curcumin concentrations and * p value < 0.05 within groups
The size of the particles was within the range of 382.2 to 1358 nm, and 956.77 to 2781.33 nm for the blank and curcumin loaded particles respectively. The size of the curcumin-loaded particles was significantly increased (P < 0.05) over the blank particles in almost all formulations. Figure 17 shows the size distribution (by intensity), graphs of blank particles, and optimised curcumin-alginate-chitosan-PF127 particles.

PDI is an indicator of the dispersity of the particles and reveals if the particles were monodisperse or not. The optimum and desirable particles are the monodispersed, which have a narrow size distribution (Azevedo et al. 2014). The PDI values ranged from 0.38 to 1 with lower ones confirming more narrow distribution.

![Size Distribution by Intensity](image)
Figure 17. Size distribution of intensity for curcumin particles a: empty particles (blank) b: formulation with 20% methanol rinse, c: formulation with 50% methanol rinse
Encapsulation efficiency is a crucial factor in the efficient characterisation of an encapsulated drug. The highest level of encapsulation efficiency was 84.29%, which was reached at a PF127 concentration of 1% (w/v). The encapsulation efficiencies in the optimal conditions were 75.69% and 72.69%, being considerably better than the encapsulation efficiency reported by Das et al. (2010), which was approximately 13%. Therefore, optimisation of different formulations under different conditions resulted in a notably higher encapsulation efficiency percentage.

The optimised formulations were selected based on the studied parameters, including encapsulation efficiency, particle size, polydispersity and zeta potential. Since the results did not reveal any significant changes in different concentrations of curcumin, the formulations with the relatively minimum size, minimum PDI and a zeta potential in the appropriate range were selected for further experiments.

From the 12 formulations, formulations 9 and 10 were selected as the optimal formulations for further testing based on their relative lower PDI, higher encapsulation efficiency and a reasonably smaller size over other formulations. However, the zeta potential was slightly lower than the acceptable range.

### 3.1.5. Morphological characterisation of the particles

TEM images (Figure 18), confirmed rod-shaped morphology of the particles contrary to most of the published reports, which have verified a spherical shape of the particles (Das et al. 2010, Bernela et al. 2014, Fattahpour et al. 2015, Bhunchu et al. 2015).

The particle size data obtained by TEM were in agreement with the data achieved from particle size analyser and the expected slight differences can be attributed to the inability of
the particle size analyser in distinguishing between the sample and the impurities. In addition, the images revealed that the overall shape of the particles was not affected by curcumin incorporation. However, they became aggregated compared to the blank particles, which supported previous zeta potential results.

![TEM images of different formulations](image)

**Figure 18.** TEM images of a: curcumin+ NaAlg+CaCl$_2$+chitosan+PF127, b: curcumin+ NaAlg+CaCl$_2$+chitosan, c: NaAlg+CaCl$_2$+ chitosan+PF127 (blank), d: NaAlg+CaCl$_2$+ chitosan (blank)

### 3.1.6. Release kinetics

*In vitro* release studies were carried out on two formulations with different concentrations of methanol rinse wash, which were selected based on their efficient level of encapsulation, along with acceptable PDI, particle size and an acceptable zeta potential. The release results for both formulated curcumin particles are given in Figure 19.

According to the observed curcumin release profiles from both formulations, curcumin release occurred in a biphasic pattern over 5 days. The statistical comparison of the above-mentioned curcumin cumulative release values achieved from different concentrations of methanol rinse (20%, and 50%) at specific times, revealed significant differences in both
formulations. Interestingly during the first 5 hours, both formulations displayed very similar release patterns with an initial burst of around 60%. However, after 7 hours the formulation with 50% methanol rinse showed a significant increase in cumulative curcumin release. After 24 hours, the release percentage in formulations with 20% and 50% methanol rinse reached 78% and 65% respectively. By the end of 96 hours, both formulations reached a cumulative curcumin release of over 90% with 50% methanol rinse formulation being dominant.

Therefore, these results, showed an initial burst corresponding to around 60% of curcumin for both formulations, which was followed by a sustained curcumin release to a total of approximately 91% and 95% for both formulations respectively. The 20% methanol rinse formulation exhibited slower and more sustained release compared to 50% methanol rinse formulation (Figure 19). The visualised rapid initial release of curcumin could be attributed to the dissociation of curcumin, which was absorbed by the surface of the polymeric matrix. However, the sustained release phase could be due to the release of curcumin entrapped inside the particle matrix.

![Release curves of curcumin particles](image)

*Figure 19. Release curves of curcumin particles. All values represent mean ± SD (n =3), and *p value < 0.05*
In order to interpret the mechanism of curcumin release, the achieved release profiles of curcumin for both formulations were fitted to five classical models including Zero order, First order, Hixon-Crowell, Higuchi, and Korsmeyer-Peppas models (Li et al. 2008, Korsmeyer et al. 1983, Fattahpour et al. 2015, Subramanian et al. 2014). The mathematical formulas for the mathematical models are as below:

1. Zero order model  \( \frac{M_t}{M_\infty} = Kt \)
2. First order model  \( \log \frac{M_t}{M_\infty} = Kt \)
3. Higuchi model  \( \frac{M_t}{M_\infty} = Kt^{1/2} \)
4. Korsmeyer-Peppas (Power law) model  \( \log (\frac{M_t}{M_\infty}) = \log K + n \log t \)
5. Hixon-Crowell model  \( (\frac{M_t}{M_\infty})^{1/3} = Kt \)

where, \( \frac{M_t}{M_\infty} \) refers to the fraction of released drug at time \( t \). The value of \( K \) and \( n \) represent the release constant and release exponent.

The observed fitted graphs are depicted in Figures 20 and 21.
Figure 20. Release kinetics of optimised 20% methanol rinse formulation
Figure 21. Release kinetics of optimised 50% methanol rinse formulation
Table 1 indicates the values for release constants (k), release exponents (n), and determination coefficients (R^2), which were calculated based on the release data fitted to the corresponding models. The best-fitted model was determined based on the determination coefficient (R^2) obtained for the mentioned kinetic models.

**Table 1.** Estimated parameters for encapsulated curcumin obtained after fitting the release experimental data to five kinetic models

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Curcumin release kinetic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero order</td>
</tr>
<tr>
<td></td>
<td>K</td>
</tr>
<tr>
<td>20% methanol wash</td>
<td>0.517</td>
</tr>
<tr>
<td>50% methanol wash</td>
<td>0.573</td>
</tr>
</tbody>
</table>

The first order profile represented the substantially higher R^2 value of 0.904 and 0.912 for 20% and 50% methanol wash formulations respectively compared to the Zero order model which gave values of 0.600 and 0.602 respectively for both formulations. This indicated that curcumin release depended on the concentration of curcumin entrapped in the particles.

The values of R^2 for Higuchi and Hixon-Crowell kinetic models were comparatively moderate. The highest R^2 value for both formulations corresponded to Korsmeyer-Peppas model, representing 0.9515 and 0.942 for 20% and 50% methanol wash formulations. Hence the achieved R^2 values for the different models revealed that the Korsmeyer-Peppas model fitted best with the curcumin release kinetic data. This finding was in agreement with the
study by Das et al. (2010). Furthermore, the value of $n$ (release exponent) was 0.1414 and 0.1987 respectively for the mentioned formulations.

The value of $n$, which indicates the mechanism of release, can reveal whether the mechanism is Fickian or non-Fickian. According to the previous reports, $n \leq 0.43$ corresponds to the Fickian behaviour (case I transport), while $n \geq 0.85$ shows a non-Fickian case II transport (relaxation-controlled behaviour) and $0.43 \leq n \leq 0.85$ represents a non-Fickian release (anomalous transport) (Takka et al. 1998, Siepmann and Peppas 2012, Peppas and Sahlin 1989, Ritger and Peppas 1987).

The obtained value for $n$, implied that the mechanism involved in curcumin release exhibited Fickian kinetics, which was mainly the result of drug diffusion across a chemical gradient. However, Das et al. (2010), reported an $n$ value of 0.84, which implied a combination of “diffusion controlled” and “swelling release” mechanisms. The $K$ value indicated the release speed, i.e. the higher $K$ value corresponded to the faster release. Therefore, as can be seen in Table 1, the studied formulations displayed $K$ values of 1.668, and 1.595, which were almost similar, with 20% methanol rinse formulation being relatively dominant.

### 3.2. Cellular experiments

These experiments were carried out to indicate the toxicity of curcumin particles, as well as empty particles on FEP-188 keratinocyte cells, along with cellular internalisation of the particles to the cells.
3.2.1. *In vitro* validation of particle cytotoxicity

3.2.1.1. Effect of particles on FEP-188 keratinocyte cell viability

The IC50 of free curcumin was 14, 11, and 7 µg/ml after 1, 3 and 5 days respectively. As most of the released curcumin (90%) was already released by the end of the third day, IC50 of 11 µg/ml was considered as the IC50 for the cell toxicity assay (Figure 22). However, encapsulated curcumin did not exhibit any significant decrease in cell viability up to 25 µg/ml of the applied concentration. Two possible explanations exist for this finding. One possibility was that polymeric alginate and chitosan still maintained interactions with released curcumin on the particle surface, decreasing its toxicity to the cells compared to the free curcumin. A second possibility was the failure of the curcumin particles to penetrate the cells, probably as a result of the low zeta potential, which lead to aggregation of the particles. Therefore, the particles could not be up taken efficiently by the cells. To further investigate the cytotoxic effect of curcumin particles, a trial including micrograph imaging was conducted with different concentrations of curcumin particles and free curcumin dissolved in DMSO on FEP-188 keratinocyte cells.

![Figure 22. Cell toxicity curve of curcumin in DMSO, curcumin particles, free particles, and control. All values represent mean ± SD (n=3), and *p value < 0.05](image)
3.2.1.2. Microscopic cell toxicity

An evident dose response was revealed in FEP-188 keratinocyte cells (Figure 23). The concentrations of 5 and 10 µg/ml of curcumin particles resulted in similar morphologies of the FEP-188 keratinocytes to that of the control cells. However, a slight decrease in density, along with minor morphology changes, were seen in cells treated with 10 µg/ml of curcumin in DMSO. By increasing the concentration up to 25 µg/ml, a prominent decrease in density of the cells treated with curcumin in DMSO was observed. Likewise, obvious morphological changes including cell aggregation, membrane shrinkage and detachment from the well surface occurred. Nevertheless, considering the changes in morphology, the cytotoxic effect of 25 µg/ml of curcumin particles was evidently less than the equivalent concentration of curcumin in DMSO, (Figure 23) confirming the role of the polymeric matrix in decreasing the cytotoxic effects of curcumin on FEP-188 keratinocyte cells. A substantial decrease in cell viability following the application of 50 µg/ml as the highest concentration of both curcumin dissolved in DMSO and curcumin particles was evident according to the micrographs. Other changes including a drastic decrease in density of the cells, cell detachment from the surface, jagged cell membrane, and more typically, rounding up the cells were also observed in both samples.

According to the micrographs, slight cell death started to happen in FEP-188 keratinocyte cells treated with 10 µg/ml curcumin in DMSO. However the initial morphology changes in the cells treated with curcumin particles were revealed at the concentration of 25 µg/ml. Thereby, the microscopic images were in agreement with the first hypothesis, indicating the effect of the polymeric matrix in decreasing the toxic effects of curcumin on FEP-188 keratinocyte cells.
Figure 23. Qualitative intracellular uptake of curcumin particles, and free curcumin in 0.4% DMSO by keratinocytes over 3 days.
3.2.2. Cellular uptake by confocal microscopy imaging

The results of the qualitative cellular uptake indicated that curcumin particles had the ability to penetrate the cells. Figure 24 demonstrates a panel of the monitored fluorescent images of FEP-188 keratinocyte cells incubated with curcumin particles, empty particles (blank) and free curcumin in PBS over a 10 hour period. The cells incubated with curcumin particles exhibited strong green fluorescence following the internalisation and accumulation of curcumin, whereas the control cells and the cells incubated with empty particles did not display any detectable fluorescence signal as expected. No fluorescent signal appeared in the cells treated with free curcumin in PBS and only minor fluorescence appeared from crystalline curcumin outside the cells, being unable to enter the cells. Furthermore, the time-dependent curcumin internalisation visualised from the microscopic images revealed low fluorescence intensity (mostly on cell membranes), during the initial hours, which was intensified with time and extended to the cell cytoplasm.

These results confirm that the polymer matrix played a major role in facilitating penetration of the curcumin particles into the cells since free curcumin in PBS was not accumulated in the cytosol and curcumin-cellular uptake was inefficient. However, more experiments are needed to be performed in order to establish the mechanisms of the internalisation process, which will be elucidated in future studies.
Figure 24. Confocal images of keratinocytes over 8 hours treated by a - e: curcumin particles, f: normal view of the keratinocytes, g: free curcumin in PBS, and h: empty particles.
Chapter 4: Conclusions and future works

Biocompatible and biodegradable formulations for promising curcumin delivery were successfully developed and optimised based on physicochemical studies. The optimum formulations were selected to perform cellular experiments aimed at understanding the toxicity of curcumin on cells \textit{in vitro} and the uptake of curcumin formulations. The size of the fabricated curcumin particles ranged from 1286 to 1485 nm, which were larger than the empty particles. According to previous studies, the size of the particles employed in drug delivery should be large enough to avoid their entrance into blood capillaries. However, if the size exceeds a specific range, there is a possibility of drug failure in being properly taken up by the cells (Cho et al. 2008).

The encapsulation efficiency of the optimised particles was enhanced to 75%, which was quite remarkable compared to the study by Das et al. (2010), on the similar polymeric structure (13%). The zeta potential exhibited values slightly below the stable range, which lead to partial aggregation of the particles in some cellular studies. Physical characterisation using TEM imaging demonstrated (i) the successful fabrication and encapsulation of curcumin in the polymeric matrix and (ii) the formation of rod-shaped particles.

Release studies confirmed a biphasic release composed of an initial burst, followed by a sustained release trend for curcumin particles. Among different mathematical models applied for modelling the release kinetics of curcumin, the Korsmeyer-Peppas model showed the
highest correlation coefficient for both optimised formulations. Based on the obtained mathematical values for the established models, the release kinetics of curcumin conformed to a diffusion mechanism. In addition, *in vitro* sustained release was shown to have enhanced efficacy compared to that of previously published reports as approximately 95% of curcumin was efficiently released by the end of the experimental period (Das et al. 2010).

*In vitro* cytotoxicity assays using the Cell Titre Blue® reagent, were carried out to estimate the systemic toxicity of both fabricated particles, as well as curcumin encapsulated particles. This assay was further investigated in microscopic scale to confirm nontoxicity of the applied concentration of curcumin particles below 25 µg/ml.

Furthermore, the results of the cellular uptake study validated the internalisation of curcumin particles, although in some duplicates curcumin could not penetrate the cells efficiently, which might have occurred as a result of the low zeta potential leading to aggregation, and subsequently precipitation of the particles.

Thus, the developed biocompatible and biodegradable formulations have the potential to be employed as drug delivery vehicles for curcumin. However, more experimental work is required in order to improve the physicochemical properties and to overcome the problem of low negativity of zeta potential which resulted in relative instability and precipitation of the particles and also subsequent larger particle size in some trials. Furthermore, the storage stability of the encapsulated curcumin particles needs to be evaluated since any physical instability would affect the delivery of the drug. Also, a series of wound healing experiments at cellular levels such as scratch migration assay, cell mito stress assay, and tube formation (angiogenesis test), should be performed to substantiate the ability of the preparations to accelerate wound healing and prepare these for *in vivo* animal trials. Further elucidation at
the cellular and molecular level to identify the precise mechanism of action of curcumin in different wounds including diabetic wounds are of pivotal importance to establish suitable therapies to improve wound healing. In later stages, extra validation using animal models is required to be able to be implemented as a tool in drug delivery.
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