DEVELOPMENT OF

MICROENCAPSULATION SYSTEMS FOR BOTH ORAL AND

TOPICAL DRUG DELIVERIES

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DEVELOPMENT OF MICROENCAPSULATION SYSTEMS FOR BOTH ORAL AND TOPICAL DRUG DELIVERIES

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

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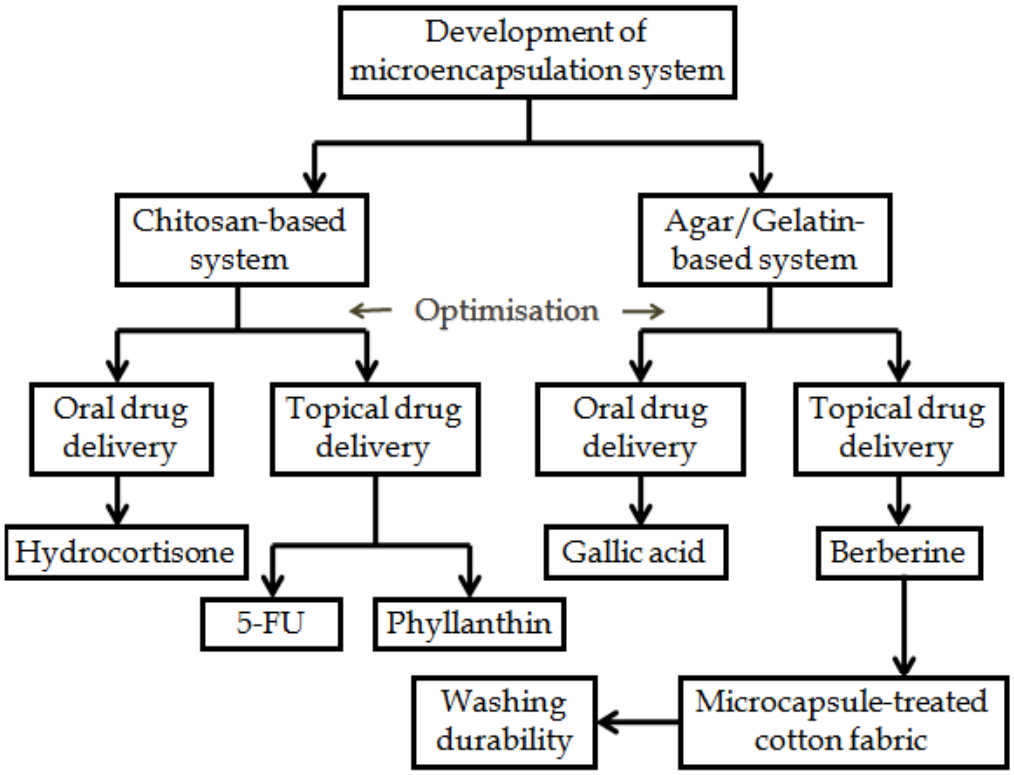
ABSTRACT

Owing to the advance in biotechnology, conventional drug dosage forms are being supplemented by versatile approaches of drug delivery. The development of both oral and transdermal drug delivery systems using microencapsulation technology would offer several possible benefits over the conventional drug delivery routes. Therefore, this thesis investigates the development, characterization and applications of microencapsulated drugs for both oral and topical deliveries.

The optimal chitosan-based microcapsules containing calendula oil were prepared by a simple coacervation method. The feasibility of both oral and topical applications of chitosan-based microcapsules was further tested using hydrocortisone succinic acid (HSA), 5-fluorouracil (5-FU) as well as phyllanthin respectively. The optimal agar/gelatin-based microcapsules were also developed to tackle the use of toxic crosslinker formaldehyde in gelatin microencapsulation system. Both oral and topical applications were demonstrated using berberine and gallic acid loaded microcapsules respectively. The development of drug containing microcapsules was demonstrated to be an efficienct deliverable tool for both oral and topical applications.

Medical textiles, the textile materials which are used for medical and healthcare applications, receive great attention in textile and clothing industries. Among various methods, microencapsulation technology is of high potential to develop the medical textiles with controlled release property of biomedical agents for different therapeutic and healthcare purposes. In this study, a chitosan-based

(oil-in-water) and an agar/gelatin-based (water-in-oil) microencapsulation systems containing berberine were applied to cotton fabrics to provide an anti-*S. aureus* activity for textile materials. After 20 washing cycles, the cotton fabrics with agar/gelatin-based microcapsules containing berberine still exhibited the anti-*S. aureus* activity. However, the chitosan-based system did not show any growth inhibition towards *S. aureus*, only the contact areas showed.



Scheme 1 Rationale of research project

LIST OF PUBLICATIONS

Referred Journal Articles

Lam PL, Li L, Yuen CWM, Gambari R, Wong RSM, Chui CH, Lam KH. Effects of multiple washing on cotton fabrics containing berberine microcapsules with anti-*Staphylococcus aureus* activity. **Journal of Microencapsulation** 2013, 30(2), 143-150.

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Lam PL, Yuen MCW, Kan CW, Wong RSM, Cheng GYM, Lam KH, Gambari R, Kok SHL, Chui CH. Development of calendula oil/chitosan microcapsules and their biological safety evaluation. **Australian Journal of Chemistry** 2012, 65(1), 72-80.

Lam PL, Gambari R, Yip J, Yuen MCW, Lam KH, Wong RSM, Wang XW, Tang JCO, Kok SHL, Chui CH. Development of phyllanthin containing microcapsules and their improved biological activity towards skin cells and staphylococcus aureus. **Bioorganic & Medicinal Chemistry Letters** 2012, 22(1), 468-471.

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Owing to the advance in biotechnology, conventional drug dosage forms and routes of administration are being supplemented by versatile and novel approaches of drug delivery. Over recent years, researchers and scientists have tended to exploit and develop innovative biotechnologies in order to overcome the limitations of traditional drug delivery systems. However, producing biopharmaceuticals with the optimal bioavailability and biostability as well as the maximum therapeutic efficiency is greatly challenged. The desired formulations should protect the drugs under unstable biological environments, such as enzymatic degradation and rapid clearance, also prolonging the delivery time to the targeted body site. In fact, many drugs present the problems of low bioavailability when they are administrated through oral and transdermal routes while some patients are found to have allergic response towards intravenous injection. There is a great potential for the development of both oral and transdermal drug delivery systems using microencapsulation technology as these offer several possible benefits over the conventional drug delivery routes. Introducing microencapsulation technology to oral and topical drug delivery systems is believed to minimize the problems in the conventional drug administration, also promoting an alternative administration route to improve the release profile and bioavailability of the drug dosage.

Since the efficacy of many drugs in conventional dosage forms, for examples: tablets and hard gelatin capsules, is often limited by their potential to reach the

targeted biological site, the need of exploiting biotechnologies and bioactive materials is fundamental to provide the desired excipient or carrier properties to achieve the targeted drug delivery. Polymeric microcapsules as oral and topical drug delivery vehicles are believed to protect the labile drugs from the external environments such as enzymatic degradation in the gastrointestinal tract (GIT), oxidation and heat. Microencapsulation, as a micro-packaging technique, refers to the manufacture of microcapsules or microspheres by surrounding the small particles of solids, droplets of liquids or dispersions of solids in liquids with thin polymer coatings to give small capsules a variety of applications. An encapsulated reservoir of core active ingredients is under the controlled release to provide useful functions for specific end-uses. The wall material is capable of protecting the active ingredients from the external environment such as acidity, alkalinity, evaporation, heat, oxidization, light or moisture. Microcapsules transfer the active ingredients for specific purposes, such as therapeutics, increasing the efficiencies and minimizing environmental damages by making use of the property of the controlled release and enabling the controlled release of active ingredients in a functional manner, for example: whether the core substance is released all at once or moderately and gradually. Microcapsules are provided within minute sizes that range from 1-1000 microns for enabling the efficiency of the controlled release (Aggarwal *et al.*, 1998; Holme, 2004; Kumar and Rane, 2004; Cheng *et al.*, 2007; Harini *et al.*, 2007; Bansode *et al.*, 2010).

In the 21st century, microencapsulation technology has been widely introduced to biomedical applications. Biodegradable polymeric microcapsules have received considerable attention as potential drug delivery devices in view of their applications in controlled release of drugs and in targeting particular body sites.

Microparticles have already been introduced to oral drug delivery systems with great success and microparticulate drug delivery models including microcapsule drug carrier systems possess high potential for various applications in therapeutic and pharmaceutical fields, such as anti-inflammatory (Saravenan *et al.*, 2003; Bayomi, 2004), antibiotics (Pandey *et al.*, 2003; Park *et al.*, 2004), anti-tumor (Sharma *et al.*, 1996; Nuijen *et al.*, 2001), proteins (Vandenberg *et al.*, 2001; Zheng *et al.*, 2004), vitamins (Shi *et al.*, 2002) and nutrition beneficial oils (Peniche *et al.*, 2004; Klinkesorn and McClements, 2009). In fact, microcapsule drug delivery systems offer many benefits to drug delivery, targeting and releasing, and their great potential to integrate with conventional drug dosages which become an effective micromedicine for oral drug delivery. The use of biodegradable, biocompatible and non-toxic polymeric materials as drug carriers in microencapsulation systems is possible to improve the drug stability in biological environments including the gastrointestinal tract (GIT), increase the drug entrapment efficiency, and utilize drug targeting, release, absorption and interaction with biological sites.

However, few researchers have studied the topical or transdermal applications of microcapsules or microparticles by applying the encapsulated drugs to the skin. In fact, conventional topical or transdermal drug deliveries have some limitations, such as slow penetration rates, lack of dosage flexibility or precision and difficulty in limiting drug uptake. Therefore, it is worthwhile to integrate microencapsulation technology with topical drug delivery in order to provide a controlled release manner for drug delivery. The advantages of tropically applied microcapsules include the minimization of inconvenience and risk during the oral and intravenous administrations, extension of drug effect at a lower dosage level

and the easier drug administration resulting in better patient compliance (Elliott, 2003; Krousel-Wood *et al.*, 2005; Ma *et al.*, 2009).

This study aims at investigating the possibility of microencapsulated drugs for both oral administration and topical application. Chitosan was used as a wall shell polymer to develop an Oil-in-Water (O/W) microencapsulation system whereas agar and gelatin were employed as the wall matrix material to develop a Water-in-Oil (W/O) microencapsulation system for both oral and transdermal drug deliveries. The use of polymeric microcapsules to encapsulate the drugs for both oral and topical usages was believed to be helpful in drug deliveries. Microencapsulated drugs were proposed to be gradually released under control from microcapsules and the sustained release could promote a longer lasting effect of core active drugs. It is speculated that chitosan-based and agar/gelatin-based microcapsules could be a versatile drug delivery vehicle for both oral and topical medications.

1.2 Objectives

The objectives of the present study are as follow:

- To develop the optimal chitosan-based (Oil-in-Water) and agar/gelatin-based (Water-in-Oil) microencapsulation systems without the use of harmful and toxic materials for both oral and topical drug deliveries with the aid of statistical analysis.
- To study the physical properties of the developed drug containing microcapsules in terms of drug loading, drug loading efficiency, particle size, surface morphology, chemical compositions as well as *in vitro* release profile of core active agents.

- To investigate the performance of some oral administrated and topical applied drugs on the developed chitosan-based and agar/gelatin-based microencapsulation systems.
- To study the biological responses and pharmaceutical activity of the drugs containing chitosan-based and agar/gelatin-based microcapsules for oral drug delivery by *in vivo* animal tests using C57BL/6 mice.
- To examine the biological activity and pharmaceutical effect of the drugs containing chitosan-based and agar/gelatin-based microcapsules for topical drug delivery by *in vitro* human keratinocyte HaCaT skin cells, human skin fibroblasts, as well as *Staphylococcus aureus* (*S. aureus*).
- To establish the nude mice skin delivery model for investigating the transdermal delivery of topical applied drug containing microcapsules.
- To evaluate the washing durability and antibacterial activity of the drugs containing microcapsule-treated cotton fabrics prepared with chitosan-based and agar/gelatin-based systems by AATCC Test Method and antibacterial assessment respectively.

1.3 Scopes of Study

The scopes of this study are to understand the development and the potential applications of chitosan-based and agar/gelatin-based microencapsulation systems for both oral and topical drug deliveries. Different drugs were entrapped into the two microencapsulation systems for oral administration and transdermal usage. The scopes of the present work are shown as follows:

- The influences of different condition parameters for production of chitosan-based microcapsules, such as chitosan concentration, types of carrier oils, ratios of core material to wall polymer, stirring speed and the

pH value on the encapsulation efficiency and particle size of developed microcapsules.

- The effects of different condition parameters for agar/gelatin-based microcapsule formation such as the ratio of agar to gelatin, the ratio of polymer to oil, the ratio of oil to surfactant as well as the stirring speed on the encapsulation efficiency and particle size of the developed microcapsules.
- The possible applications of microencapsulated drugs using chitosan-based and agar/gelatin-based systems for both oral and topical drug deliveries.
- The comparison of an antibacterial activity between the drug containing chitosan-based and agar/gelatin-based microcapsule-fabricated cotton fabrics after multiple washing cycles.

1.4 Research Methodology

- Literature review was conducted to enhance the background knowledge concerning the recent development in the research areas.
- Preliminary trials were performed to develop the optimal (O/W) and (W/O) microencapsulation systems for both oral and topical drug deliveries. Careful selection of suitable wall shells and core materials was considered during the development of microencapsulation systems.
- Optimal condition parameters for the production of chitosan-based (O/W) microencapsulation system in terms of wall shell concentration, types of oils, core/wall ratio, stirring speed, and pH value were generated with the aid of statistical analysis.
- Optimal condition parameters for the production of agar/gelatin-based (W/O) microencapsulation system in terms of the ratio of agar to gelatin, the ratio of

polymers to oil, the ratio of oil to surfactant and the stirring speed were decided with the aid of statistical analysis.

- Drug loading, drug loading efficiency, particle size, surface morphology, chemical compositions and release profile of the developed drug containing microcapsules were characterized by using UV-Vis spectrophotometer, Particle Size Analyzer, Scanning Electron Microscopy (SEM) and Fourier Transform Infrared spectrophotometer (FTIR). The *in vitro* controlled release for oral administrated and transdermal applied drug containing microcapsules was studied with respect to the simulated stomach and intestinal conditions and simulated human skin condition respectively.
- Optimal chitosan-based and agar/gelatin-based microencapsulation systems were integrated with several drugs for both oral and topical drug deliveries.
- Biomedical and pharmaceutical responses as well as safety issue of the drugs containing chitosan-based and agar/gelatin-based microcapsules for oral drug delivery were examined by *in vivo* animal tests using C57BL/6 mice to analyze the effects of microcapsules in a systematic and objective way.
- Biological and pharmaceutical effect as well as secure issue of the drugs containing chitosan-based and agar/gelatin-based microcapsules for topical drug delivery were evaluated by *in vitro* human keratinocyte HaCaT skin cells, human skin fibroblasts, as well as *Staphylococcus aureus* (*S. aureus*).
- The possibility of transdermal delivery of topical applied drug containing microcapsules was demonstrated using the nude mice skin delivery model.
- The washing durability and antibacterial activity of the drugs containing microcapsule-treated cotton fabrics prepared with chitosan-based and agar/gelatin-based systems were investigated using AATCC Test Method, SEM images, FTIR analysis as well as antibacterial assessment.

- To prepare, summarize and write up the thesis.

1.5 Arrangement of Thesis

The thesis consists of eight chapters. Chapter 1 outlines the background, objectives, scopes and methodology of this study.

Chapter 2 is the literature review that provides an introduction of microencapsulation technology and the materials used to develop the microencapsulation systems in this study. In this Chapter, the research background, microencapsulation technologies, release mechanisms of core materials, applications as well as the characteristics of both wall and core materials used in the experiment are mentioned.

Chapter 3 covers the experimental details in developing the optimal chitosan-based (Oil-in-Water) microencapsulation system including the materials and apparatus used as well as procedures of developing an O/W microencapsulation system using a simple coacervation technique. This chapter mainly focuses on the development of optimal condition parameters for production of chitosan-based microcapsules in terms of concentration of chitosan solution, types of carrier oils, ratios of core material to wall polymer, stirring speed and the pH value. The oil loading efficiency, particle size, surface morphology, chemical compositions, oil release profile as well as *in vitro* and *in vivo* toxicity of the optimal chitosan-based microcapsules are investigated.

Chapter 4 shows the use of the optimal chitosan-based microencapsulation system to encapsulate the hydrocortisone succinic acid (HSA) into chitosan wall

shell for oral drug delivery. The physical properties of HSA containing chitosan microcapsules including drug loading efficiency, particle size, surface morphology, chemical compositions and oil release profile are evaluated. The pharmacological activity of HSA containing chitosan microcapsules is examined using the plasma adrenocorticotropic hormone (ACTH) of C57BL/6 mice.

Chapter 5 discusses the use of the optimal chitosan-based microencapsulation system to encapsulate 5-fluorouracil (5-FU) and phyllanthin into chitosan wall shell so as to enhance the inhibition of 5-FU towards excessive keratinocyte growth and the antioxidant property of phyllanthin towards human fibroblast cells and skin keratinocytes as well as its antimicrobial activity towards *Staphylococcus aureus*. The physical properties of microcapsules including drug loading efficiency, particle size, surface morphology, chemical compositions and oil release profile are examined. The developed microcapsules containing 5-FU and phyllanthin are suggested to be useful for topical applications.

Chapter 6 demonstrates the development of an optimal agar/gelatin-based (Water-in-Oil) microencapsulation system without the use of toxic and harmful crosslinker – formaldehyde. Agar together with gelatin as the wall matrix materials are employed to prepare the microencapsulation system by the crosslinking method, and focus to investigate the optimal condition parameters of producing agar/gelatin-based microcapsules containing berberine and gallic acid in terms of the ratio of agar to gelatin, the ratio of polymer to oil, the ratio of oil to surfactant and the stirring speed. The determination of an optimal condition for producing drugs containing agar/gelatin microcapsules as the drug delivery model is mainly based on the drug loading efficiency (first priority) and the mean

particle size (second priority). Afterwards, berberine and gallic acid loaded agar/gelatin microcapsules are used to demonstrate the feasibility of the developed microencapsulation system for both topical and oral drug deliveries. The drug loading efficiency, particle size, surface morphology, chemical compositions and drug release profile of the optimal drug containing microcapsules are investigated. The optimal berberine loaded microcapsules are examined by the *Staphylococcus aureus* (*S. aureus*) assay and nude mice skin delivery model while the gallic acid containing microcapsules are evaluated by a mice disease model.

Chapter 7 is to discuss berberine to be used as a model agent to be encapsulated into both chitosan-based (oil in water) and agar-gelatin (water-in-oil) microencapsulation systems to demonstrate the antibacterial activity of these two systems after various washing cycles. The SEM images, chemical compositions and anti- *S. aureus* activity of the washed samples are evaluated.

Chapter 8 provides a general conclusion of the present thesis. Recommendations for further study are also proposed in this chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Owing to the advance in biotechnology, conventional drug dosage forms and routes of administration are being supplemented by versatile and novel approaches of drug delivery. Over recent years, researchers and scientists have tended to exploit and develop innovative biotechnologies in order to overcome the limitations of traditional drug delivery systems. However, producing biopharmaceuticals with the optimal bioavailability and biostability as well as the maximum therapeutic efficiency is greatly challenged. The desired formulations should protect the drugs under unstable biological environments, such as enzymatic degradation and rapid clearance, also prolonging the delivery time to the targeted body site.

Some patients are found to have allergic response towards intravenous injection. There is a great potential for the development of both oral and transdermal drug delivery systems using microencapsulation technology as they offer several possible benefits over the conventional drug delivery routes. They involve the minimization of inconvenience and risk during intravenous administrations, extension of drug effect at a lower dosage level and the easier drug administration resulting in better patient compliance. As a result, the exploitation of the micron drug delivery model for both oral administrations and topical applications integrated with medical textiles is believed to overcome the limitations of conventional drug delivery systems.

2.2 Common Routes of Drug Delivery and Their Limits

There are several common routes of drug administration, involving oral, pulmonary, transdermal/topical (through the skin), transmucosal (nasal, buccal, ocular) and parenteral (injection) paths (Pork, 1996; Davenport, 1998; Patel, 2007; Ravi Kumar, 2008).

Oral delivery is the most attractive, convenient and cost saving route for drug administration. However, many drugs, especially peptide, protein and gene-based drugs are not easy to pass through mucosal surfaces and biological membranes, susceptible to enzymatic degradation within the gastrointestinal tracts or not able to be effectively absorbed into the systematic circulation because of their molecular size and charge issues (Pork, 1996; Davenport, 1998; Patel, 2007; Ravi Kumar, 2008).

Pulmonary delivery is responsible for the treatment of respiratory diseases. However, the pulmonary delivery for some drugs, such as proteins, is instable under the presence of proteases in the lung, resulting in low bioavailability, and suffered by the barriers between capillary blood and alveolar air (Patel, 2007).

Transdermal/topical delivery is to apply the drugs to the skin, the drawbacks of oral administration including gastrointestinal degradation and rapid clearance in the liver, therefore, can be avoided. However, the limitations still exist. They include slow penetration rates, lack of dosage flexibility or precision and difficulty in limiting drug uptake (Patel, 2007).

In transmucosal drug delivery, drugs are absorbed via a mucosal surface, the

stratum corneum epidermidis, the major barrier to drug absorption across the skin is absent, resulting in fast transport of drugs to the systematic circulation. Although transmucosal delivery is able to solve the problems presenting in oral and transdermal drug delivery, the mucosa surfaces as the site for drug delivery present the disadvantages as well. A major drawback of transmucosal drug administration is the lack of dosage form from retention at the site of absorption if other than the low flux associated with musocal delivery (Davenport,1998).

Parenteral delivery also preferred to intravenous, intramuscular and subcutaneous injections. The advantages of parenteral drug delivery involve the prevention of gastrointestinal degradation and first pass hepatic metabolism in oral drug delivery, as well as the difficult access of drugs to brain, ocular and intra-articular cavities (Davenport, 1998). Most protein-based drugs are administrated by injection nowadays. However, drug delivery by injection also contains some problems. The insertion site in skin by an injection needle may carry a risk of infection or allergy, resulting in redness, pain and discomfort. The patients also suffer from distress and inconvenience when parenteral administration is carried out. Hypothermia may occur when large amount of cold injected fluids is accessed to the human body (Ravi Kumar, 2008).

2.3 Microencapsulation

2.3.1 Definition of Microencapsulation

Microencapsulation, as a micro-packaging technique, refers to the manufacture of microcapsules or microspheres by surrounding the small particles of solids, droplets of liquids or dispersions of solids in liquids with thin polymer coatings to give small capsules a variety of applications. An encapsulated reservoir of core

active ingredients is under the controlled release to provide useful functions for specific end-uses. The wall material is capable of protecting the active ingredients from the external environment. Microcapsules are provided within minute sizes that range from 1-1000 microns for enabling the efficiency of the controlled release (Aggarwal *et al.*, 1998; Palmieri *et al.*, 2002; Holme, 2004; Kumar and Rane, 2004; Nii and Ishii, 2005; Cheng *et al.*, 2007; Harini *et al.*, 2007; Bansode *et al.*, 2010; Petrovic *et al.*, 2010).

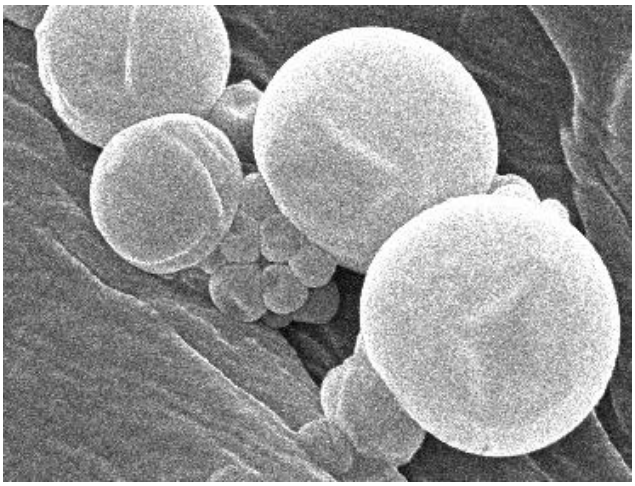


Figure 2.1 SEM image of microcapsules

Microcapsules can be produced by an oil-in-water system and a water-in-oil system. Generally, an oil-in-water system refers to the processing emulsion which the oil is used as a dispersed phase while the water-soluble/hydrophilic material is employed as a dispersed medium during microcapsule formation process. However, a water-in-oil system means that the processing emulsion contains the water-soluble/hydrophilic material as the dispersed phase and the oil as the dispersed medium in the microcapsule formation process. Figure 2.2 shows the schematic diagrams of an oil-in-water system and a water-in-oil system. For the potential applications of an oil-in-water and a water-in-oil

systems, an oil-in-water system could be applied to the deliveries of oil-soluble agents, including fat-soluble vitamin A, D, E and K, nutrient beneficial oils as well as insoluble drugs (e.g. celebrex). A water-in-oil system could be applied to the deliveries of oil-soluble agents, including water-soluble vitamin B and C, antibiotics, chemotherapeutic drugs (e.g. cisplatin).

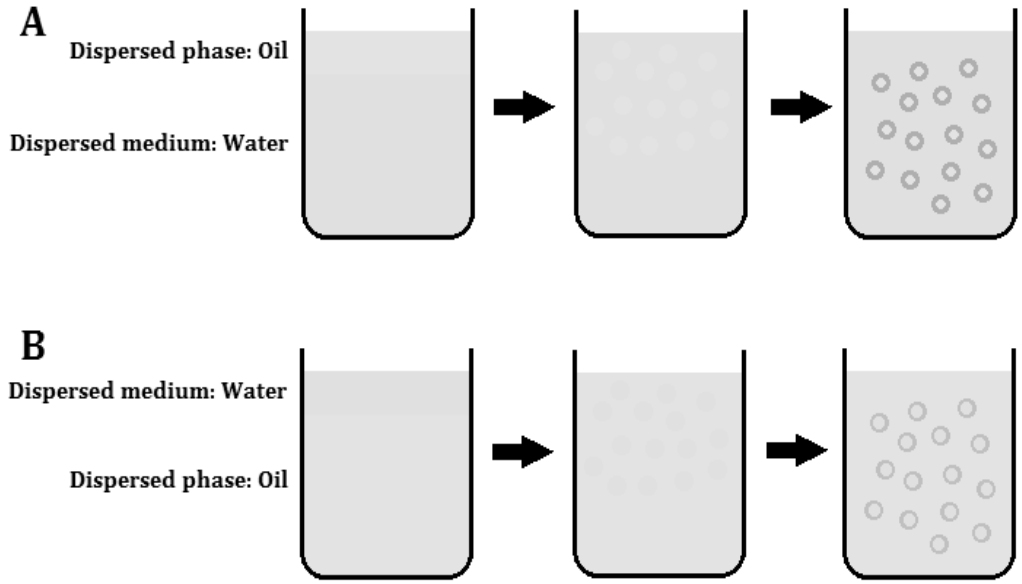


Figure 2.2 Schematic diagrams of (A) an oil-in-water system and (B) a water-in-oil system

2.3.2 Background of Microencapsulation Technology

The earliest concept of microencapsulation could be traced back to 1930s by the use of spray-drying method (Simon, 2006; Cheng *et al.*, 2007) and the introduction of "coacervation" by Dutch Scientists Bungenberg de Jong and Kruyt (Benita, 2006). Until 1950s, the initial application of microencapsulation technology was carbonless copy papers, which were developed by Barrett Green of the National Cash Register (NCR) company. The carbonless papers were produced by the use of coacervation technique (Simon, 2006; Cheng *et al.*,

2007).

After the invention of carbonless papers, the microencapsulation technology was further developed by the US-based Eurand America (Erkan and Sariisik, 2004) in order to create novel and versatile applications in various aspects. Until 1970s, the first invention of microencapsulation technology in textiles was broken out by introducing the microencapsulated dyes, pigments, softeners, anti-static agents and fire retardants for textiles. The next invention was in 1990s which carried thermochromic and photochromic materials, anti-microbials and insect repellents (Boh and Knez, 2006).

Over recent years, microencapsulation technology has integrated with various fields including drug deliveries, biomedical functions, climate-control properties, cosmetic effects, aromatherapy, fragrances and phase change materials have been found (Simon, 2006; Cheng *et al.*, 2007).

2.3.3 Advantages of Applying Microencapsulation Technology in Oral and Topical Drug Deliveries

By using the microencapsulation technology, several benefits can be found in both oral and topical drug deliveries (Aggarwal *et al.*, 1998; Holme, 2004; Kumar and Rane, 2004; Cheng *et al.*, 2007; Harini *et al.*, 2007; Bansode *et al.*, 2010):

(1) Protecting the core active ingredients such as liable drugs, from external environments such as acidity, alkalinity, evaporation, heat, oxidization, light or moisture;

(2) Improving the handling of drugs during processing;

(3) Improving the workability as liquids can be prepared into powder or solid forms, making materials work easily;

(4) Enabling the controlled and sustained release of active ingredients or compounds in a functional manner whether the core drug is released all at once or moderately and gradually;

(5) Transferring the active ingredients for specific purposes, such as pharmacology and therapy;

(6) Improving drug bioavailability; and

(7) Increasing efficiencies and minimizing environmental damages by working use of the property of the controlled release.

2.3.4 Structural Details of Microcapsules

The structure of a microcapsule is composed of a core active substance that is encapsulated within a polymer coating or a wall shell material (Aggarwal *et al.*, 1998; Holme, 2004; Kumar and Rane, 2004; Cheng *et al.*, 2007; Harini *et al.*, 2007; Bansode *et al.*, 2010). Microcapsules generally consist of two main components which are core material and wall material. The basic structure of a microcapsule is shown in Figure 2.3:

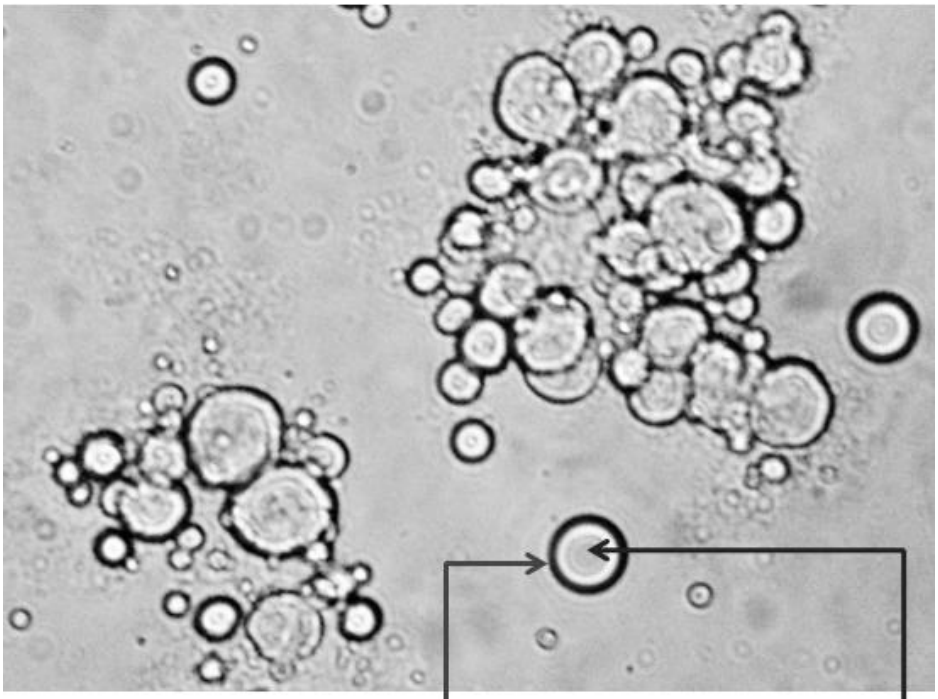


Figure 2.3 Basic structure of a microcapsule

(1) Active ingredients (Core material)

Active ingredients are the substance in form of liquid, solid or gas, such as solvents, fragrances, and drugs (Kumar and Rane, 2004). It is also termed to core contents, internal phase, active, encapsulate, payload or fill (Cheng *et al.*, 2007). They are encapsulated into the polymer shell and are released under control.

(2) Polymer shell (Wall material)

A polymer shell that surrounds the active ingredients is also called the wall, shell, external phase, membrane or matrix. A variety of natural sources has been shown to become the potential non toxic, biodegradable, biocompatible and mucoadhesive wall polymers. Table 2.1 indicates the different categories of wall materials used in microencapsulation which are classified as follows (Sachan *et al.*, 2006):

Table 2.1 Categories of wall materials

Vegetable Gums	Gum Arabic, agar, sodium alginate, carrageenan and dextran sulphate
Celluloses	Ethyl cellulose, nitrocellulose, carboxy methylcellulose, cellulose acetate phthalate and cellulose acetate butyrated phthalate
Proteins	Collagen, gelatin, casein, fibrinogen, hemoglobin and poly amino acids
Condensation polymers	Teflon, nylon, polymethane, polycarbonate, amino resins, alkyl resins and silicone resins
Homopolymer	Poly vinyl chloride, polyethylene, polystyrene, poly vinyl acetate and poly vinyl alcohol
Copolymers	Maleic anhydride copolymer with ethylene or vinyl methyl ether, acrylic acid copolymers and methacrylic acid co-polymers (eudragit)
Curable polymers	Epoxy resins, nitro paraffin and nitrated polystyrene
Waxes	Wax, paraffin, rosin, shellac, tristerium, monoglyceride, bees wax, oils, fats and hardened oils
Other	Chitin and chitosan

The physical appearance of the microcapsule is governed by the physical property of the core material (Holme, 2003). The microcapsule may be irregularly shaped due to the original shape of the core particle if solids or crystalines are encapsulated as shown in Figure 2.4A. Simple spherical microcapsules can also be obtained if a liquid or gas is entrapped as shown in Figure 2.4B. Further structural diversity may be obtained in the form of aggregated microcapsules. The microcapsules may exhibit a multi-core structure in which multiple core droplets are enclosed within one polymer coating as shown in Figure 2.4C.

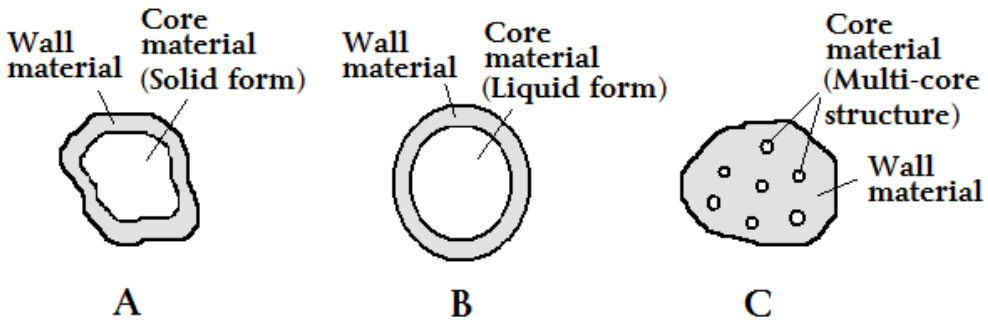


Figure 2.4 Schematic diagrams for three types of microcapsules: (A) Irregular-shaped microcapsule; (B) Spherical microcapsule; (C) Multi-nuclear microcapsule. (A) and (B) are the reservoir type, (C) is the matrix type

Microcapsules are typically produced in a wide range of sizes with diameters being in the range of 1 to 1000 microns. However, capsules may be made with diameters between 0.01 and 10,000 microns in theory (Anon, 2005). The wall thickness of a microcapsule is generally between 0.5 and 150 microns but the wall thickness of less than 0.5 microns can also be achieved. The core content of the microcapsule can be between 20% – 95% of the total mass (Kumar and Rane, 2004).

2.3.5 General Review of Microencapsulation Techniques

Microencapsulation can be achieved by many different methods based on the physical properties of the core material. Core material is classified into the forms of solids, liquids and gases. Each of them is achieved by the use of different microencapsulation processes as given in Table 2.2 (Sudha *et al.*, 2005; Benita, 2006; Cheng *et al.*, 2007).

Table 2.2 Microencapsulation processes for specific forms of core materials

Types of Methods	Microencapsulation Processes	Forms of Core Materials
Physical	Air-suspension	Solids, powders
	Centrifugal extrusion	Solids, liquids
	Fluid bed coating	Gases
	Spray drying	Solids, liquids
Chemical	Emulsion hardening	Liquids
	In-situ polymerisation	Solids, liquids
	Interfacial polymerisation	Solids, liquids
	Phase separation (simple and complex coacervation)	Solids, liquids
	Polymer-Polymer Incompatibility	Solids

(1) Coacervation (phase separation)

Coacervation refers to the phase separation in a macromolecular system in which two phases are formed (Sovilj *et al.*, 2010). In the process of coacervation, the core material is first suspended or dispersed in a solution of wall material before any droplet formation steps. The particles produced with size within range of 2μm to 1200μm with respect to variations in different dispersing parameters,

such as stirring speed, stirring time and viscosity. Coacervation starts with a change of pH value of the dispersion by adding a coacervating agent, resulting in a precipitation of wall material and a continuous coating of wall polymer around the core droplets (Bansode *et al.*, 2010). Phase separation in simple coacervation is achieved by reducing the solubility of a polymer through changing the temperature, adding non-solvent or salting out by electrolytes while complex coacervation requires the introduction of another oppositely charged macromolecules (Bansode *et al.*, 2010; Sovilj *et al.*, 2010). Simple coacervation requires only a single colloidal solute while complex coacervation involves more than one colloid (Aggarwal *et al.*, 1998). Figure 2.5 shows the formation of microcapsules by complex coacervation.

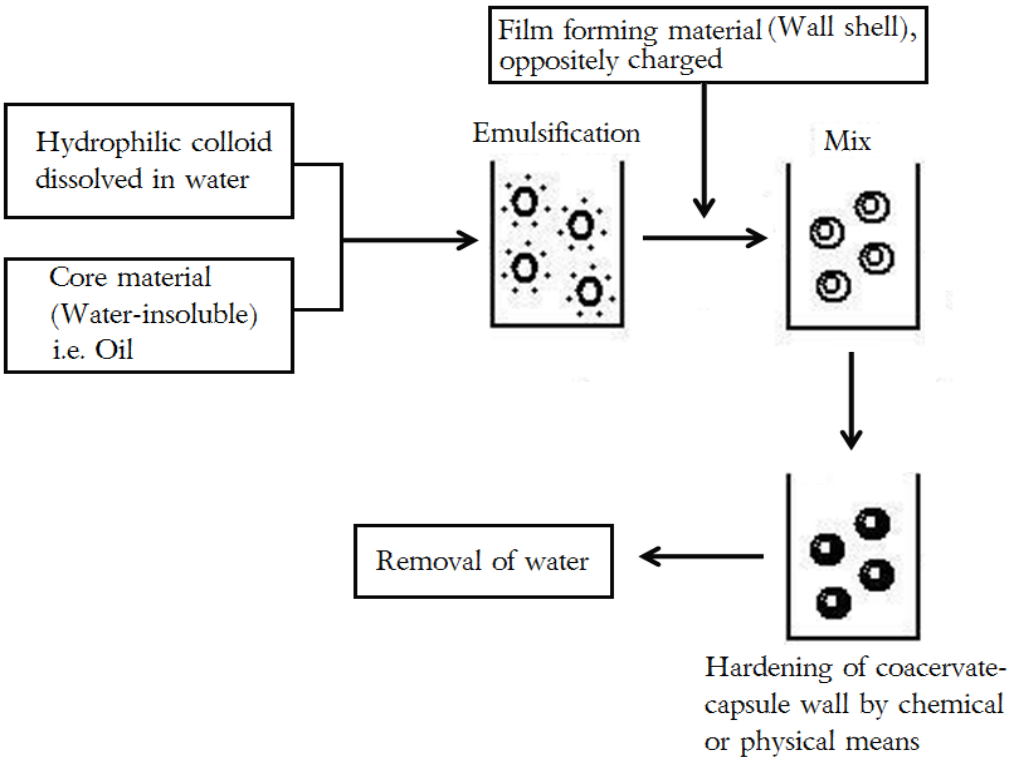


Figure 2.5 Schematic diagram showing the formation of microcapsules of oil droplets in water by complex coacervation (Redrawn from Cheng *et al.*, 2007)

(2) Emulsification solvent removal technique

In this method, wall polymers are first dissolved in a low water miscible and volatile organic solvent, and the core material is dispersed in the polymer solution. The mixture is then emulsified in an aqueous phase, resulting in oil-in-water emulsion. Afterwards, the emulsified mixture is evaporated or extracted for producing microspheres. The micro-particles are washed, collected by filtration and dried, and the free-flowing injectable microspheres are obtained (Benita, 2006).

(3) Spray-drying

The spray-drying method was industrially employed since 1927 (Peniche *et al.*, 2003). In spray-drying process, the core particles are firstly dispersed in a wall polymer solution and then sprayed into a hot chamber, as shown in Figure 2.6. The wall material solidifies onto the core particles because the input solvent evaporates and therefore the microcapsules can be formed in a polynuclear or matrix type (Jyothi *et al.*, 2010). Spray-drying is normally used for encapsulating labile material such as fragrance due to its short contact time in the drier (Sudha *et al.*, 2005; Cheng *et al.*, 2007). Lorenzo-Lamosa *et al.* developed chitosan-based microsphere for colonic delivery of sodium diclofenac by using the spray-drying technique (Peniche *et al.*, 2003).

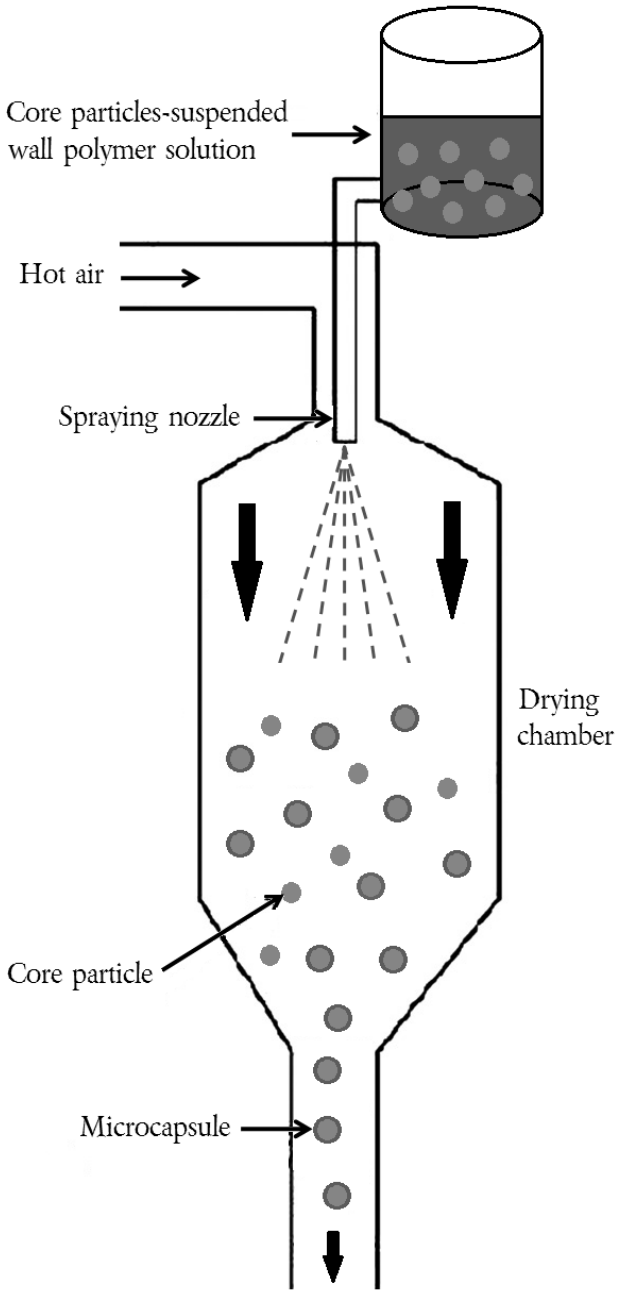


Figure 2.6 Schematic diagram showing the formation of microcapsules by spray-drying (Redrawn from Jyothi *et al.*, 2010)

(4) Centrifugal extrusion

Liquid forms of the core material are entrapped within the wall polymer solution by using a rotating extrusion head with concentric nozzles (Sudha *et al.*, 2005; Cheng *et al.*, 2007). The core material flows into the pre-formed membrane of

wall material, leading to the extrusion of a rod of material (Cheng *et al.*, 2007). Droplets break away from the rod and become hardened by heating, cooling or solvent evaporation in order to form the solid microcapsules. This method is suitable for producing microcapsules with liquid or slurry as the core material (Sudha *et al.*, 2005; Cheng *et al.*, 2007). Figure 2.7 shows the diagram of centrifuge two-fluid nozzle used for producing microcapsules.

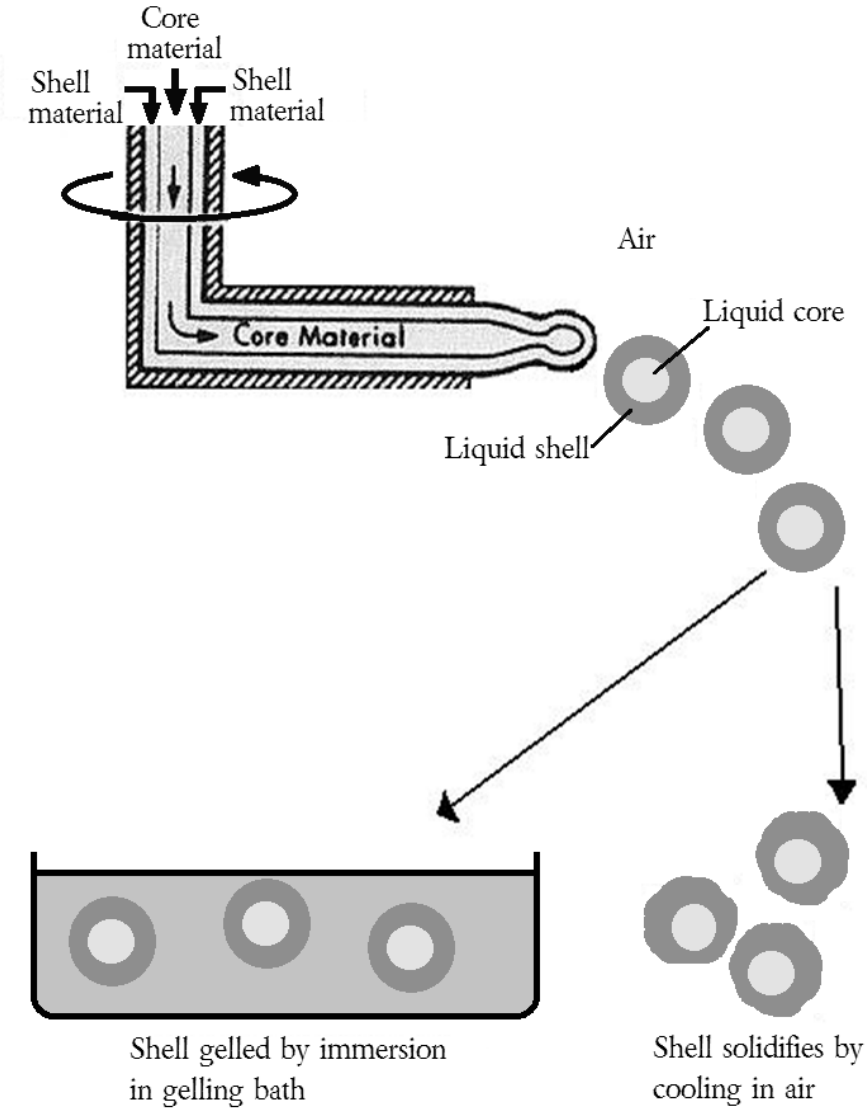


Figure 2.7 Schematic diagram of centrifuge two-fluid fozzle used for producing microcapsules (Redrawn from Cheng *et al.*, 2007)

(5) Air suspension

The core particles are coated by the dissolved polymers while being suspended on an upward moving air steam in the coating chamber. In the chamber, the cyclic flow of particles pass through the coating zone portion of chamber, where the coating polymer solution is sprayed onto the particles (Sudha *et al.*, 2005; Cheng *et al.*, 2007). The coating is hardened due to the solvent evaporation or cooling of a melt (Cheng *et al.*, 2007). The cycles are repeated until the full encapsulation of core particles and the desired thickness are achieved. This process is only suitable for the encapsulation of solid forms of core materials (Sudha *et al.*, 2005; Cheng *et al.*, 2007).

(6) Interfacial polymerisation and in-situ polymerisation

As for the Interfacial polymerisation, the wall matrix of microcapsules is formed at or on the surface of a droplet or particle by the reactive monomer polymerisation. A multi-functional monomer is dissolved in the liquid form of core materials, and the mixture is dispersed to a desired drop size in an aqueous phase involving a dispersing agent and a multi-functional amine. The quick reaction of polymerisation then produces the wall matrix of microcapsules (Cheng *et al.*, 2007). In the in-situ polymerisation, polymerisation happens in the continuous phase and on the continuous phase side of the interface, and the pre-polymer with lower molecular weight generates there. No reactive agents are included in the core material. The pre-polymer coats onto the dispersed core material being encapsulated where polymerisation constantly happens, resulting in the production of a solid wall matrix of the microcapsules (Cheng *et al.*, 2007).

(7) Crosslinking

Crosslinking refers to the formation of chemical links between molecular chains to produce a three-dimensional network of the connected molecules (Jyothi *et al.*, 2010). The strategy of covalent crosslinking has been applied in several commerical technologies and scientific interests so as to improve the characteristics of the resulting polymer system or interface, such as thermosets and coatings (Stevens, 1999, Wicks *et al.*, 1999). The degree and density of crosslinking as well as the molecular structures can affect the swelling property of the crosslinked system. The most commonly employed crosslinker is formaldehyde in the gelatin–based microencapsulation system. However, the wall shell or wall matrix of microcapsules made from formaldehyde is more rigid. Apart from formaldehyde, it was also reported the use of glycerol as a crosslinking agent in the gelatin-gum Arabic microencapsulation process (Huang *et al.*, 2007).

2.3.6 Considerations of Selecting the Microencapsulation Techniques

Many techniques can be used for entrapping solids, liquids and gases as the core content of microcapsules. The following factors are considered to be the selection criteria for the selection of microencapsulation techniques (Nelson, 2001; Boh and Knez, 2006; Madene, 2006):

(1) Properties of core materials and wall polymers used in the system, such as the solubility of materials;

(2) The reactivity between wall polymers and core substances;

(3) The appropriate concentration of wall polymers and core materials present in the microencapsulation system;

(4) The functionality of the final microcapsules;

(5) Range of particle sizes, wall thickness and wall permeability of microcapsules;

(6) Types and release rates of core ingredients; and

(7) Physical properties and economical consideration of manufacturing the microencapsulated finishes.

2.3.7 Release of Core Material from Microcapsules

2.3.7.1 Release of Core Material

In the microencapsulation system, the release of a core material embedded in a polymer shell is actually the ability to deliver an active ingredient over an extended period of time to a certain target in order to achieve specific purposes. The core substances may be released either gradually through the wall shells, that is known as the controlled release or diffusion, or when the external environments such as friction, pressure and change of temperature trigger the dissolution of the polymer walls, or by biodegradation (Cheng *et al.*, 2007). The core ingredients can be released all at once while the polymer shells burst under outside pressure, melt under heat, burn under high temperature and degrade under light influences (Kan *et al.*, 2005). No matter what factors trigger the release of core materials, the terminal goal of microencapsulation is to achieve the controlled release of the core substances in order to extend the release period of the active ingredients to meet the specific purposes of the system.

2.3.7.2 Controlled Release of Microcapsules

2.3.7.2.1 Definition of Control Release

Controlled release is a percentage zero order release such that the core material releases over time irrespective of concentration. In another words, it is the ability

of a delivery system to release one or more active ingredients over an extended period of time at a controlled rate (Madene, 2006).

2.3.7.2.2 Mechanisms of Drug Release

The core drugs can be gradually released through the polymer shells of microcapsules by several methods:

(1) Diffusion of core substances through a wall shell or wall matrix

This mechanism is commonly related to the process wherein the dissolution fluid penetrates the wall of microcapsules and then dissolves the core drugs. Finally, the dissolved drug leaks out through the holes in the wall shell or wall matrix (Korsmeyer *et al.*, 1983). Disffusion is affected by (1) the rate at which dissolution fluid penetrates the wall of microcapsules, (2) the rate at which drug dissolves in the dissolution fluid, and (3) the rate at which the dissolved drug leaks out and disperse from the surface (Gunder *et al.*, 1995). Figure 2.8 shows the schematic diagram of diffusion of a core drug.

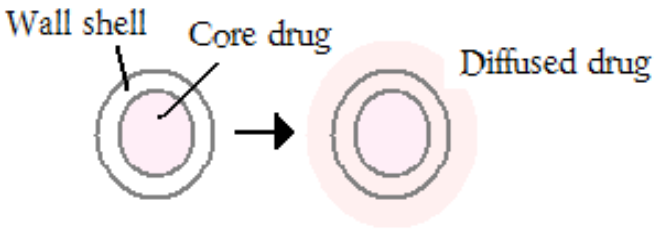


Figure 2.8 Schematic diagram of diffusion of a core drug

(2) Release of core substances through chemical reactions

The core drugs may be released by the chemical stimulus, as the polymer walls are eroded or cleaved through the chemical reactions between the wall shells'

compounds and the external chemicals' compounds (Madene, 2006).

(3) Solvent activation of the polymer shell

Since the polymer used for wall material is solvent-activated and placed in a thermodynamically compatible medium, the shell polymer swells and the core drug is released as the swelling behavior of the polymer wall allows the core ingredient to migrate more easily. The degree of swelling for a polymer wall is controlled by water absorption or the presence of solvents such as glycerine or propylene glycerol as shown in Figure 2.9 (Madene, 2006).

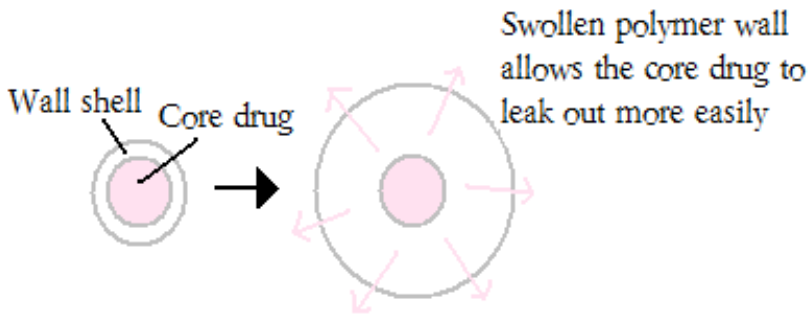


Figure 2.9 Schematic diagram of solvent activation of a polymer wall

2.3.7.2.3 Factors Affecting the Rate of Controlled Release

Rate of the controlled release can be affected by the following three main factors:

(1) Properties of wall materials

The properties of polymer walls largely affect the rate of controlled release. The structures of wall shell or wall matrix are influenced by the conditions of preparation. Polymer wall characteristics such as crystallinity, crosslink density, thickness and porosity may largely determine the controlled release rate of the core drugs of microcapsules. Increase in crystallinity and crosslink density of the

polymer shell indicates the lower release rate.

(2) Properties of the core materials

The characteristics of core active substances including molecular weight, solubility, disffusibility, chemical functionality, polarity and relative volatility to wall shell material properties, as well as the parameters of encapsulation technology can also affect the drug release rate of the microcapsules.

(3) External environments

The core ingredients may be released by mechanical stimulus including friction, pressure, as well as chemical stimulus and thermal stimulus such as heat.

2.3.7.2.4 Advantages of Controlled Release

There are several benefits of the controlled release which include (Sachan *et al.*, 2006):

(1) Prolonged release dosage from

The microencapsulated drugs can be administrated orally as the microcapsule is perhaps helpful for prolonged drug release as compared with conventional tablet and capsule forms.

(2) Absorption in targeted body sites

Microencapsulation technology can be employed to produce the enteric-coated dosage forms. Therefore, the medicine will be selectively absorbed in the intestine rather than the stomach.

(3) Aid in mechanical aspects

Oily medicines can be encapsulated into the tableted dosage forms with the aid of microencapsulation technology. Non-flowable drugs including the non-flowable multicomponent solid mixture of niacin, riboflavin, and thiamine hydrochloride and iron phosphate may also be entrapped and made into tablets using microencapsulation process.

(4) Drug protection

Microencapsulation can provide a polymer barrier for drugs which degrades in the existence of heat, moisture, oxygen and light. For example, vitamin A and K have been demonstrated to be protected from the presence of moisture and oxygen via microencapsulation.

(5) Simple handle of noxious substances

Microencapsulation can be applied to greatly reduce the potential danger of handling of the toxic and poisonous substances including pesticides, herbicides and pesticides.

(6) Others

The use of core drugs may be decreased due to the prolonged release of microencapsulated medicines. Many drugs have been microencapsulated in order to reduce the volatile and hygroscopic properties. Some medicines have been entrapped into microcapsules to decrease gastric irritation during oral administration.

2.3.7.2.5 Challenges of Controlled Release

Although the controlled release brings the significant advantages, there are several challenges faced by the microencapsulation industry that involve:

(1) Difficulties in developing release formulations for water-insoluble drugs

Challenges exist in developing controlled release formulations for drugs with poor water solubility, which need both solubilization and engineering of release profile. A controlled drug delivery may be required to achieve the prolonged exposure or time-based release for a water-insoluble drug under certain circumstances. In fact, a direct use of current coating-based or matrix-based delivery systems without technology fabrication will fail to accomplish an acceptable controlled release of a water-insoluble drug.

(2) Challenges in preparing the formulations for drugs with low bioavailability

Difficulties are present in preparing the formulations for drugs with low bioavailability such as peptides and proteins. These drugs are susceptible to enzymatic degradation within the gastro-intestinal tract and poor absorbed when they are oral-administrated.

(3) High cost

The relatively high cost of formulation designs occurs in the excipient preparation, processes and equipments in order to help to achieve desired release profiles for drugs with different attributes.

(4) Others

Producing biopharmaceuticals with the optimal bioavailability and biostability as well as the maximum therapeutic efficiency is greatly challenged. The controlled

release systems with biodegradable, biocompatible and non-toxic properties are also highly preferable.

2.3.7.3 Microencapsulation Techniques and their Release Mechanisms

Different microencapsulation methods perform the controlled release behavior through different mechanisms. Table 2.3 shows different microencapsulation techniques and their release mechanisms (Madene, 2006).

Table 2.3 Microencapsulation techniques and their release mechanisms

Microencapsulation technique	Controlled release mechanism
Simple coacervation	Prolonged release
Complex coacervation	Prolonged release (diffusion) and started release (pH, dehydration, mechanical, dissolution or enzymatic
Spray drying	Prolonged release and started release
Fluid bed drying	Started release (pH or heat treatment)
Extrusion	Prolonged release

2.3.8 Evaluation of Performance of Microcapsules

To study the microcapsules and the performance of microcapsule-treated textiles, there are many devices available for providing the detailed evaluation of microcapsules. Tables 2.4 and 2.5 show the common evaluation tools of microcapsules and microcapsule-treated textiles (Choi *et al.*, 2004; Zhu *et al.*, 2005 ; Gupta *et al.*, 2006; Kin *et al.*, 2006 ; Abdel-Fattah *et al.*, 2007; Ji *et al.*, 2007; Salaun *et al.*, 2008; Shanmuganathan *et al.*, 2008).

Table 2.4 Common evaluation tools of microcapsules

Evaluation Tools	**Characteristics examined**
Fourier Transform Infrared Spectrophotometer (FTIR)	Chemical ingredients identification of microcapsules
Particle Size Analyzer	Size in diameters and size distribution of microcapsules
Zeta Potential Analyzer	Zeta Potentials of microcapsules
Scanning Electron Microscope (SEM)	Morphology of microcapsules
High Performance Liquid Chromatography (HPLC)	Amount of core active ingredients
Inverted Microscope	Shape of microcapsule shells
UV – VIS Spectrophotometer	Control release of microcapsules and the amount of core substance entrapped in the microcapsules
Franz Diffusion Model Finite Dosage Apparatus	*In vitro* release of microcapsules
Differential Scanning Calorimeter (DSC)	Thermal storage and release properties of microcapsules
Thermal Analyzer	Thermogravimetic analysis (TGA) of microcapsules
Mercury intrusion Porosimeter	The median pore size and porosity of 3-D microcapsules

Table 2.5 Common evaluation tools of microcapsule-treated textiles

Evaluation Tools	**Characteristics examined**
Scanning Electron Microscope (SEM)	Surface morphology of microcapsule-treated fabrics
Fourier Transform Infrared Spectrophotometer (FTIR)	Chemical ingredients identification of microcapsule- treated fabrics
Automatic Washing Machine	Laundering durability of microcapsule-treated fabrics

2.3.9 Applications of Microencapsulation

2.3.9.1 Microencapsulation in Biomedical Applications

In the 21st century, microencapsulation technology has been widely introduced to biomedical applications. Biodegradable polymeric microcapsules involving chitosan-based microcapsules have received considerable attention as potential drug delivery devices in view of their applications in the controlled release of drugs and in targeting particular organs or tissues (Patel, 2007). Researchers and scientists considered the microencapsulation system as a way of storing medicinal substances at microscopic level and providing the subsequent release of core materials. Nowadays, in the pharmaceutical fields, the major interest of microencapsulation is currently in the application of anti-inflammatory (Saravenan *et al.*, 2003; Bayomi, 2004), antibiotics (Pandey *et al.*, 2003; Park *et al.*, 2004), anti-tumor (Sharma *et al.*, 1996; Nuijen *et al.*, 2001) and proteins (Vandenberg *et al.*, 2001; Anal *et al.*, 2003; Zheng *et al.*, 2004; Chen *et al.*, 2009). In health and medicinal applications, microencapsulation systems associated with vitamins (Shi and Tan, 2002) and nutrition beneficial oils (Peniche *et al.*, 2004; Klinkesorn and McClements, 2009) have also been discussed.

2.3.9.2 Microencapsulation in Biomedical Textile Applications

Medical textile is one of the most rapidly expanding fields in textiles and clothing industry. With the increasing consideration of healthcare towards the customers, textile manufacturers tend to exploit novel technologies in producing the medical textiles in order to satisfy the customers' needs. There is a great potential to encapsulate the medical ingredients into the textiles for particular suffers, such as acetaminophen for pain killing, steroid for anti-inflammation with the use of microencapsulation technology. One particular example is to

impart tamoxifen microcapsules into cotton fabrics of clothing for treating the breast cancer in the daily dressing of patients (Ma *et al.*, 2009). The possibility of encapsulating drugs for the controlled release to provide regular medication is one stimulant application. The active drug agents of the medical textiles are released under friction and pressure during human wear, people can then experience the medical treatment via a simple daily wear.

Nowadays, many patients from the clinical wards need to receive intra-venous injection of therapeutic drugs. However, some of them are sensitive to intra-venous injection. Unfortunately, many drugs are not suitable to be taken orally because of the low bioavailability and gastro-intestinal absorption problems. The development of the microcapsules related biomaterials and biotextiles may solve these issues by attaching them on the skin and allow diffusion of the therapeutic drugs through the skin layer into the peripheral circulation. Furthermore, the development of the microcapsules related medical textiles can be used as an alternative form of oral administrated carriers. This provides an improvement in the time-control-release efficiency. The biocompatibility of medical agent delivery system should also be recognized in the development of medical textiles. Biocompatibility is referred to the ability of a material to behave its desired properties in specific applications without exhibiting any harmful effects towards human body. As a result, the medical textiles should not elicit any toxicity and stimuli to the skin during the release of active agents.

2.3.9.3 Microencapsulation in Other Textile Applications

Recently, Microencapsulation technology has been applied in the textile industry. The most popular uses involve (1) phase change materials; (2) fragrance finishes and aromatherapy (3) polychromic and thermochromic finishes; (4) flame retardant finishes; (5) antimicrobial and antiodor finishes; (6) inspect repellent finishes and (7) skin care finishes and (Nelson, 2001; Nelson, 2002; Holme, 2003; Holme, 2004; Kumar and Rane, 2004; Erkan and Sariisik, 2004; Wang *et al.*, 2009). However, little work has been done on the possibility of development of biomedical textiles with the aid of microencapsulation technology.

(1) Phase change materials

United States (US) National Aeronautics and Space Administration (NASA) encapsulated phase-change materials (PCMs) in space suits in order to reduce the extreme temperature variations encountered by astronauts in the early 1980s. Outlast has also imparted this technology into textile clothing such as vests, snowsulits and trousers; and into the household items such as blankets, mattresses and pillowcases. In 1998, Accordis, in Bradford, United Kingdom (UK), has incorporated the Outlast microcapsules into textile fibres without damaging their nature, such as softness, strength and drape.

(2) Fragrance finishes and aromatherapy

Through microencapsulation, fragrances can be maintained on textile garments. Microencapsulation of essential oil flavors was been applied to children's clothing. In 1979, R T Dodge of Dayton in Ohio, produced the microencapsulated 'scrath and sniff' T-shirts and women's hosiery, and it was claimed that their products can survive after 8-20 washing cycles. Celessence

International of Hatch End in Middlesex, produced the microencapsulated fragrant-smelling merchandises, initially in paper products and now in textiles. The Matsui Shikiso Chemical Company of Kyoto investigated a method of fixing aroma compounds to fabrics using microcapsules. The Eldorado International Company of Seoul developed the aroma-treated fabrics that could overcome up to 25 washing cycles.

(3) Polychromic and therochromic finishes

There are two major types of color-changing systems, thermochromatic that changes color in response to temperature and photochromatic that alters color in response to UV light. Some companies applied the microencapsulated dyes to textile garments so that the color of garments could be changed in response to human heat. Microencapsulated thermochromatic dyes could survive up to 20 laundering cycles.

(4) Flame retardant finishes

Flame retardant properties can be imparted into textile merchandises via the use of microencapsulation technology. This technology has been used in military applications, such as tentage. Microencapsulated flame retardant compounds during spinning of polyester fibres for blending with cotton is one of the examples of flame retardant finishes.

(5) Antimicrobial and antiodor finishes

Microencapsulated antimicrobial and antiodor ingredients can be applied to textile apparel such as underwear, sport wear, socks and gloves, and to household textiles such as carpets, curtains and cushions.

(6) Counterfeiting finishes

Microencapsulation can be applied to a covert yet distinctive marking system in order to solve illegal copying problems. For example, in 1999, Gundjian and Kuruvilla of Nocopi Technologies developed microcapsules with a color former or an activator which were then applied to garments' labels and threads.

(7) Skin care finishes

Cosmetic textile becomes an interesting investigation tool on skin care, especially with garments encapsulated with skin moisturizing agents and skin cooling formulations for use after exposure to sunlight. Textile clothing with the controlled release of vitamins and pro-vitamins has been developed in order to offer skin care and well-being. Encapsulated anti-aging reagents have also been imparted into garments so as to reduce cellulite production by maintaining the skin elasticity.

(8) Others

Other microencapsulation applications include encapsulated insect-repellent or insect-resistant treatments on textiles; chemical protection finishes on military garments via microencapsulation technology and anti-UV finishes on apparel.

2.3.10 Criteria of Attractive Medical Textiles

There are four main criteria for an attractive medical textile:

(1) Good influencing power such that the textile products should promote good biomedical and pharmaceutical effects or functions;

(2) Good stability such that the biomedical properties should not be weakened by storage conditions;

(3) High durability such that the biomedical agent releasing properties of the textile products should be long lasting or extending when used; and

(4) High retention such that the biomedical ingredients in products should be capable of surviving after being diluted or dispersed in water during washing or laundering and finally adhered to the textile products.

2.4 Chitosan-based Microencapsulation System

2.4.1 Chitosan as a Wall Polymer for Microencapsulation

2.4.1.1 Characterization of Chitosan

Chitosan is a cationic linear polysaccharide composed of β(1-4) linked glucosamine with *N*-acetylgucosamine. It is obtained from the extensive deacetylation of chitin presented in the shells of crustaceans. There are different obtaining procedures that can affect the soluble properties of chitosan (Remunán-López *et al.*, 1998; El-Gibaly, 2002; Peniche *et al.*, 2003; Sunil *et al.*, 2004; Mohamed *et al.*, 2005; Wang *et al.*, 2006; Mortazavian *et al.*, 2007; Sui *et al.*, 2008; Pedro *et al.*, 2009). Non-soluble chitosan can be obtained by a heterogeneous procedure while water-soluble chitosan can be produced by homogeneous deacetylation of chitin and the acetylation of highly deacetylated chitosan (Peniche *et al.*, 2003). Chitosan is mostly used for being a coat or shell, but not a capsule because it has comparatively low efficiency for increasing viability of probiotic cells (Mortazavian *et al.*, 2007). Chitosan must be dissolved in the media with acidic pH, and using 1-3% aqueous acetic acid solution to solubilize chitosan, and it is very common. This is because chitosan containing the amino group which is water-insoluble is only positively charged and soluble at the state of pH<6 (Peniche *et al.*, 2003; Yang *et al.*, 2004; Sunil *et al.*, 2004; Mohamed *et al.*, 2005; Mortazavian *et al.*, 2007; Pedro *et al.*, 2009). Figure 2.10

shows the chemical structure of chitosan.

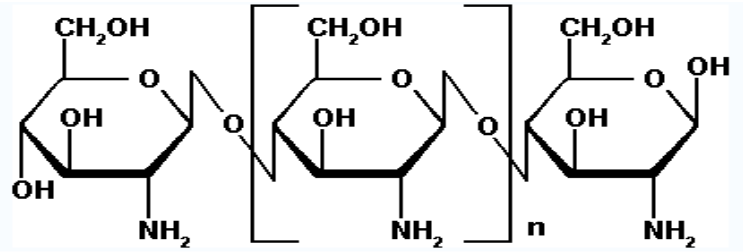


Figure 2.10 Chemical structure of chitosan

Chitosan has many advantages in producing microcapsules. Chitosan is a natural polymer which is biodegradable; biocompatible; non-toxic and safe. It is capable of controlling the release of active substances and avoiding the use of hazardous organic solvents during particle fabrications because of its solubility merely in aqueous acidic media. Chitosan is readily available for cross-linking as it contains free amino groups. It is cationic allowing the ionic cross-linking with multivalent anions. It is amenable to chemical modification. It also has an excellent film-forming ability (Peniche *et al.*, 2003; Sunil *et al.*, 2004; Mohamed *et al.*, 2005).

Chitosan is mucoadhesive and positively charged that increases residual time at the site of adsorption and prolongs the contact time between positively charged chitosan and negatively charged mucin in cell membrane, and thereby improving the drug bioavailability (Tiyaboonchai, 2003). Chitosan mucoadhesion was proposed to significantly increase the half time of its clearance (Soane *et al.*, 1999). A higher degree of deacetylation (>65%) can increase the charge density, and eventually improve the drug transpostation (Tiyaboonchai, 2003). Chitosan is considered as a safe material for microcapsule-based oral drug delivery

carriers that protect the liable drug from the gastrointestinal environment.

However, chitosan also has some drawbacks in developing microencapsulation systems. Chitosan has high viscosity and it has low solubility at pH values higher than 7.4 because of its weak base of pKa 6.2-7. Its immuno-stimulating properties can be affected by molecular weight, degree of deacetylation, purity and route of administration (Mohamed *et al.*, 2005).

2.4.1.2. Application of Chitosan

As for the application in drug delivery system, chitosan is used as binder, disintegrant and coating material. In protein delivery system, chitosan has been confirmed to be capable of improving the mucosal absorption of peptides. In addition, chitosan solutions and powers have been proved to enhance the immune response to antigens such as influenza and pertussis. Chitosan is now effectively being applied to the non-viral gene delivery systems for mucosal application (Mohamed *et al.*, 2005). With regard to drug delivery for oral cavity treatments, chitosan can be used for films or gel entrapping drugs such as Nystatin for treating oral mucosuitis (Pedro *et al.*, 2009). Table 2.6 shows various biomedical applications of chitosan-based microencapsulation systems.

Table 2.6 Biomedical applications of chitosan-based microencapsulation system

Chitosan-based microcapsules	Core substances	Biomedical applications	References
Chitosan microcapsules	Grapefruit seed extract	Antimicrobial properties and pleasant fragrance delivery	Alonso *et al.*, 2010
Genipin-crosslinked polymeric alginate-chitosan microcapsules	BSA	As a potential delivery vehicle for biomedicals	Chen *et al.*, 2009
Chitosan microcapsules	Celecoxib	A safety model for oral drug delivery	Cheng *et al.*, 2010
6-Oxychitin-chitosan microcapsules	Miconazole	Optimisation of 6-Oxychitin-Chitosan microcapsule system	Genta *et al.*, 2003
Chitosan/calcium alginate microcapsules	Nitrofurantoin	Study on intestinal delivery	Hari *et al.*, 1996
Chitosan matrix cross-linked with glutaldehyde microcapsules	Astaxanthin	Improved heat stability	Higuera-Ciapara *et al.*, 2004
O/W chitosan microcapsule system	Volatile citronella oil	Mood lifting	Hsieh *et al.*, 2006
Genipin-crosslinked chitosan-alginate complex	Indomethancin	Drug delivery model with a good cellular compatibility	Mi *et al.*, 2002
Genipin-crosslinked chitosan hydrogels	/	Wound dressing	Muzzarelli *et al.*, 2009
Chitosan/calcium alginate-based capsules	Shark liver oil	Convenient uses in therapy and prevention oils from oxidation	Peniche *et al.*, 2004
Calcium alginate/chitosan microcapsules	Vibrio bacterin	Fish vaccination	Polk *et al.*, 1994
Chitosan microsphere-templated microcasules	Heparin	Effective system of heparin delivery	Shao *et al.*, 2009
Chitosan/ethyl-cellulose complex microcapsules	Vitamin C and Vitamin D2	Improvement in drug loading (>86%) and prolonged release in intestine condition	Shi *et al.*, 2002
Chitosan/chondroitin sulfate complex microcapsules	Low molecular weight heparin (LMWH)	Drug release study	Sudha *et al.*, 2005
Alginate-chitosan core shell microcapsules	Beta-galactosidase	Improved enzyme stability	Taqieddin *et al.*, 2004
Alginate-chitosan coacervate microcapsules	BSA	*In vitro* release study	Vandenberg *et al.*, 2001
Hollow chitosan-alginate multilayer microcapsules	Doxorubicin	*In vitro* and *in vivo* studies for tumor cell investigation	Zhao *et al.*, 2007
Alginate-chitosan-poly (lactic-co-glycolic acid) (PLGA) composite	BSA	*In vitro* release study	Zheng *et al.*, 2004

2.5 Agar-Gelatin based Microencapsulation System

2.5.1 Agar-Gelatin as the Wall Polymers for Microencapsulation

2.5.1.1 Characterization of Agar

Agar is a natural polysaccharide extracted from seaweed, mainly composed of agarose and agaropectin. Agarose is a predominant component of agar composed of the repeating monomeric unit of agarobiose whereas agaropectin is a charged component of agar which consists of D-galactose and 3,6-anhydro-L-galactopyranose and monoesterified sulfuric acid units. Agaropectin is a heterogeneous mixture of smaller molecules that occur in lesser amounts. Agarose gives a high gel-forming property whereas agaropectin offers a very weak or no gel-forming property. The ratio of agarose to agaropectin is based on the species and the habitation area of the original seaweed or the extracting process (Miyazawa *et al.*, 2000). Agar molecules are present in random coils when they are dissolved in hot water at high temperature (>85˚C), while they are supposed to form double helices, which subsequently crosslink together with gelatin molecules to give a gel network at low temperature (<30˚C). Figure 2.11 shows the chemical structure of agar.

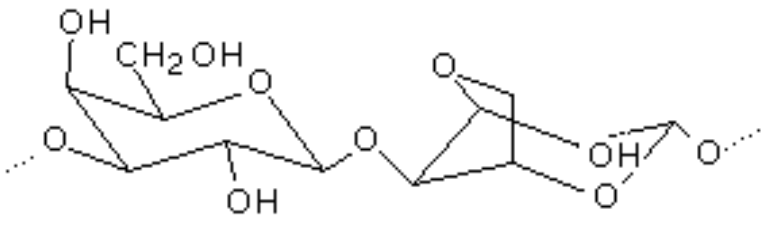


Figure 2.11 Chemical structure of agar

2.5.1.2 Characterization of Gelatin

Gelatin is a derived protein substance from collagen which is isolated from the boiled connective tissues, tendons, bones and skins of animals, usually fishes, cows and pigs (Kim *et al.*, 2006). Gelatin is regarded as a natural polymer which is positively charged, biodegradable and biocompatible. Apart from tryptophan and low in methionine, cystine and tyrosine, all the amino acids exist in gelatin (Jamilah and Harvinder, 2002). The amino acid composition in gelatin varies from one source to another and mainly composed of great amounts of glycine, proline and hydroxyproline (Gilsenan and Ross-Murphy, 2000). Gelatin is a gelling agent that is soluble in hot water and subsequently capable of forming strong, transparentelastic and elastic thermoreversible gels and flexible films on cooling below about 35℃. Generally, there are two types of gelatins, Type A and Type B. Type A gelatin is produced by soaking skin or bones in a diluted acidic medium followed by extraction at acidic pH while Type B gelatin is solubilized at near neutral pH at about 60-90℃ (Arvanitoyanni, 2002). Figure 2.12 shows the chemical structure of gelatin.

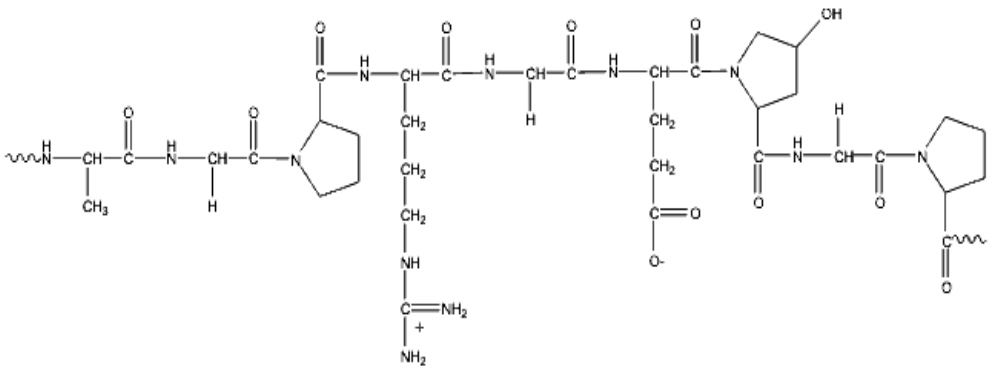


Figure 2.12 Chemical structure of gelatin

2.5.2 Core Materials

2.5.2.1 Calendula Oil

Calendula is safe to use in medicine and food for many year. Its product - calendula infused oils contains vitamin A, carotene, allantoin and flavonoids are beneficial to human health. In medical fields, researchers found that an aqueous extract of *Calendula officinalis* obtained showed non-cytotoxic activity and anti-tumor activity in mice (Kemper, 1999; Ukiya *et al.*, 2006; Jimenez-Medina *et al.*, 2006). Some *in vivo* studies also state that calendula gently stimulates the immune system and promotes lymphatic drainage, reducing inflammation and pain, lowering cholesterol and triglycerides (Mayes, 2001). The herbal mixture containing aqueous extract of *Calendula officinalis* was proved that no significant toxicological effects for oral administration (Roopashree *et al.*, 2009).

2.5.2.2 Almond Oil

Almond oils are obtained from seeds of *Prunus Amygdalus Dulcis*. It contains glucosides, minerals, vitamins A, B1, B2, B6 and E; oleic acid and linoleic acid. It is also rich in protein and fatty acids. It is very similar in composition to olive oils and good for all skin types, helpful to relieve itching, soreness, dryness and inflammation (Ahmad, 2010). Almond oil also helps to lower the cholesterol, prevent cancer, boost the immunity system and reduce the hepatotoxicity (Davis and Iwahashi, 2001; Ahmad, 2010).

2.5.2.3 Apricot Kernel Oil

Apricot kernel oil is cold pressed and refined from the pits of *Prunus armeniaca*, a small yellow to orange soft fruit. It consists of oleic acid, linoleic acid and unsaturated fatty acids esterfied with glycerin, minerals, vitamins A, B1, B2, B6,

C, E and GLA. It assists in skin moisturizing and softening, aiding the skin in retaining elasticity, clarity and suppleness. Apricot Kernel Oil is also used as an antitussive and anti-asthmatic agent to treat tumors in traditional Chinese medicine. It aids in calming the inflammation and irritation of eczema and dermatitis (Krashen, 2009).

2.5.2.4 Olive Oil

Olive oil is cold pressed and originated from the fruits of *Olea Europaea L.* Olive tree cultivation is widely located in the Mediterranean countries and it is crucial to the agricultural economy, local heritage and environment. Olive oil is the edible vegetable oil that has been stated to have its unique therapeutic and health beneficial characteristics to human beings. Olive oil contains health valuable components including monounsaturated fatty acids, phytochemicals and phenolic compounds (Oi-Kanoa *et al.*, 2007). The active compounds present in olive oil have been shown to reduce the risks of cardiovascular diseases and high cholesterols (Visioli and Galli, 1998; Covas, 2007), to improve the responses of immune and inflammatory systems (Stark and Mader, 2002; Oi-Kanoa *et al.*, 2007) and to promote an antioxidant activity (Lavelli, 2002; Visioli *et al.*, 2002; Vissers *at al.*, 2004).

2.5.2.5 Hydrocortisone

Hydrocortisone is a synthetic form of corticosteroid administrated when the body is deficient in the natural hormone. It is used to treat allergy, inflammation, asthma, collagen diseases, adrenocortical deficiency, shock and some neoplastic conditions including acute lymphoblastic leukemia. Hydrocortisone is sensitive to heat and must not be autoclaved, so it should be prepared under sterile

conditions (Iwasaki, 1987; Rigge *et al.*, 2005; Wang *et al.*, 2009). The major clinical usages of hydrocortisone include: (1) anti-inflammation which can reduce and prevent tissue inflammation; (2) action on immune system which can reduce and prevent the cell mediated immune responses as well as allergy; (3) anti-virus and anti-coma which can reduce the body response towards viral infections, reduce cellular damages and protect the human body (Wang *et al.*, 2009). Figure 2.13 shows the chemical structure of hydrocortisone.

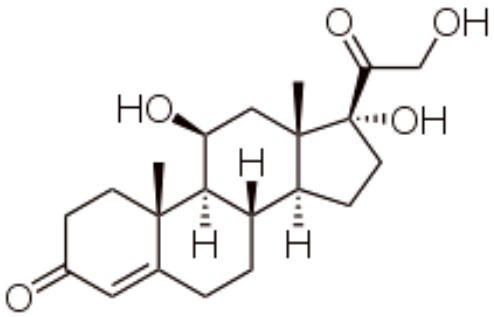


Figure 2.13 Chemical structure of hydrocortisone

In fact, many drugs are chemically modified to give more water soluble derivatives and their clinical responses will be determined by the conversion into their parental drugs. Hydrocortisone sodium succinate (HSS) is in the form of hydrocortisone ester sodium salt. It is practically more soluble in aqueous solution than hydrocortisone. Therefore, it is commonly used in aseptic formulations (Rigge *et al.*, 2005). Hydrocortisone has been reported to treat children with asthma (Iwasaki, 1987). The side effect of hydrocortisone has been proven to be reduced when using the insulin/alginate shell as a carrier for drug delivery (Zhao *et al.*, 2010).

2.5.2.6 5-Fluorouracil

5-Fluorouracil (5-fluoro-1H-pyrimidine-2, 4-dione), also known as 5-FU, is a hydrophilic cytotoxic agent used topically to treat various forms of skin pre-malignant conditions. It is specific towards skin diseases and is believed to exert little adverse effects on normal skin cells (Tsur *et al.*, 1990; Bhalla and Thami, 2003). 5-FU is also used as an effective anticancer drug for skin cancers and its active compound revealed an appropriate antitumoral activity in the topical treatment of lesions liked squamous cell carcinoma such as actinic keratosis, keratoacanthoma, Browen's disease and superficial epithetliomas of the skin (Ashbel, 1971; Paolino *et al.*, 2008). Figure 2.14 shows the chemical structure of 5-FU.

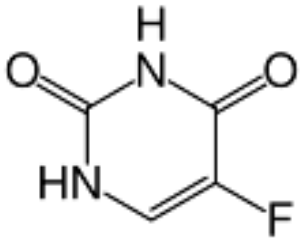


Figure 2.14 Chemical structure of 5-fluorouracil

5-FU has been reported as topical delivery systems of skin cancer (Ashbel, 1971; Paolino *et al*, 2008) and severe localized cutaneous necrosis (Bhalla and Thami, 2003). It has also been shown that 5-FU has good clinical efficacy for the topical treatment of cholesteatoma (Takahashi *et al.*, 2005).

2.5.2.7 Phyllanthin

Phyllanthin is a major bioactive lignan component isolated from the plant extract of *Phyllanthus amarus* which is a family of Euphorbiaceae (Krithika *et al.*, 2009;

Chirdchupunseree and Pramyothin, 2010; Khan *et al.*, 2010). *Phyllanthus amarus* also known as Bhui amla, is a herb which exists in central and south India, the rain forests of Amazon and other tropical areas in the world. This plant has been used in traditional medicine for many years. There has been reported that *Phyllanthus amarus* held the antimicrobial activity towards different microorganisms (Oluwafemi and Debiri, 2008; Chitravadivu *et al.*, 2009; Adegoke *et al.*, 2010). Recent studies also discussed the hepatoprotective potential of *Phyllanthus amarus* by its antioxidant activities (Sabir and Rocha, 2008; Krithika *et al.*, 2009; Chirdchupunseree and Pramyothin, 2010; Khan *et al.*, 2010). Figure 2.15 shows the chemical structure of phyllanthin.

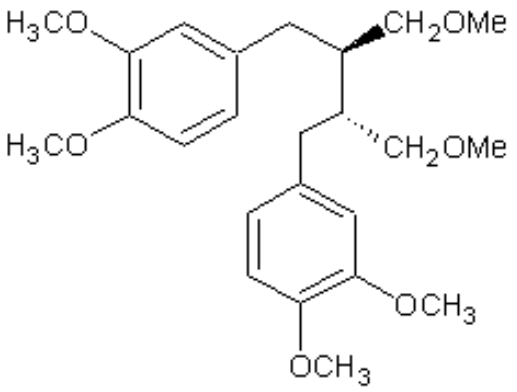


Figure 2.15 Chemical structure of phyllanthin

2.5.2.8 Berberine

Berberine is a quaternary alkaloid isolated from natural plants originated from Berberidaceae, Coptis, Fumariaceae, Mahonia, Papaveraceae and other herb species that are traditionally used Chinese herbal medicine for the treatment of gastrointestinal diseases and other infectious diseases for many years (Cernakova *et al.*, 2002; Jin *et al.*, 2010; Zhang *et al.*, 2011). Berberine HCl has also been proven to have anti-inflammatory (Choi *et al.*, 2006), antibacterial (Cernakova *et*

al., 2002; Jin *et al.*, 2010; Zhang *et al.*, 2011), antifungal (Zhao *et al.*, 2006), antitumor (Fukuda *et al.*, 1999), antioxidant (Turkoglu *et al.*, 2010) and cardiovascular (Hong *et al.*, 2003) properties. Berberine has been reported as an effective antibacterial agent against a variety of microorganisms, such as Gram-positive and Gram-negative bacteria, fungi, plasmodia and trypanosomes (Cernakova *et al.*, 2002). The antibacterial mechanism of berberine involves tightly binding to bacterial DNA duplication, RNA transcription and protein biosynthesis and affecting the enzymes which including in DNA synthesis. Berberine can also damage the bacterial cell surface structure and inhibit the growth of bacteria (Jin *et al.*, 2010). Figure 2.16 shows the chemical structure of berberine.

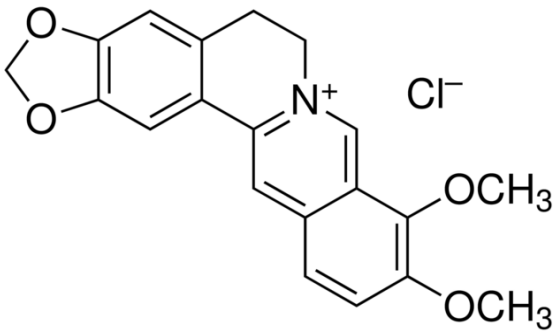


Figure 2.16 Chemical structure of berberine

2.5.2.9 Gallic Acid

Gallic acid (3, 4, 5-trihydroxybenzoic acid) is an effective natural antioxidant agent originated from the plant kingdom. It commonly exists in phenolic materials such as tannins, flavonoids and lignin that offer the plants with protection against the oxidative reaction and free radicals (Slawinska *et al.*, 2007). Gallic acid has been reported to have antibacterial (Akiyama *et al.*, 2001),

anticancer activity (Faried *et al.*, 2007), antifungal (Hussin *et al.*, 2009), antiviral (Osborne *et al.*, 1981), and antioxidant (Li *et al.*, 2005) properties as well as anti-melanogenic activity via the inhibition of tyrosinase activity (Kim, 2007).It was proposed that gallic acid exhibited the protective effect over the oxidative damage in rat liver and kidney (Rasool *et al.*, 2010; Vijaya Padma *et al.*, 2011). Figure 2.17 shows the chemical structure of gallic acid.

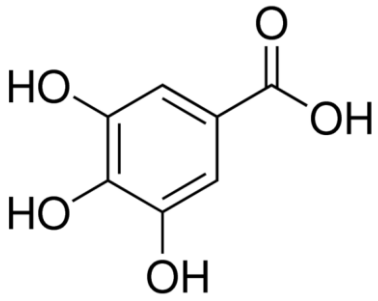


Figure 2.17 Chemical structure of gallic acid

CHAPTER 3

OPTIMIZATION OF CHITOSAN – BASED MICROENCAPSULATION SYSTEM

3.1 Introduction

Owing to the trends of healthcare all over the world, people pay more attention to invent novel biotechnologies in producing biomaterials with the aim of satisfying the users' requirements in biomedical fields. Drug delivery associated with microencapsulation technology is under consideration because microencapsulated drugs are supposed to promote comparatively effective therapeutic effects on human absorption and prolong the controlled release of drugs. In the present research, a chitosan-based microencapsulation system is developed in order to provide a safe delivery model for both oral and topical drug deliveries.

The present study attempts to use health beneficial oils in microcapsule manufacturing to prepare the chitosan-based microencapsulation system. A simple coacervation technique was used for the delivery of health beneficial oil by oral administration and topical application. Simple coacervation offers some advantages in respect to complex coacervation (Hörger, 1975). First, simple coacervation allows the use of relatively inexpensive inorganic solvents for inducing the phase separation. When complex coacervation is employed, it is necessary to use relatively expensive complex polymeric materials such as gum arabic. Simple coacervation is flexible in operation as compared to complex coacervation that is susceptible to little change in pH. The focus attends to the development of optimal condition parameters in the production of chitosan-based

microcapsules in terms of concentration of chitosan solution, types of carrier oils, ratios of core material to wall polymer, stirring speed and the pH value. The preliminary experimental results further speculated that the developed microcapsules could be used as a safe drug carrier both topically and orally.

3.2 Experimental

3.2.1 Materials

Chitosan (75%-85% deacetylated, from Aldrich, Germany) was dissolved in 1% acetic acid. Calendula oil, almond oil and apricot kernel oil were purchased from Easy Creation Asia Limited, Hong Kong. Sodium hydroxide (NaOH), acting as a hardening agent, was purchased from AnalaR, England. N-Hexane, used for microcapsules rinsing and free oil extraction, was bought from Tedia, United States. Triton X-100 purchased from Sigma, Germany was used as a dispersing agent of developed microcapsules. Pepsin from porcine gastric mucosa (Sigma-Aldrich, Germany), Trypsin from porcine pancrease (Sigma-Aldrich, Germany) and Amino Lipase A from *Aspergillus niger* (Aldrich, United States) were applied to the *in vitro* release test.

3.2.2 Preparation of Chitosan-Based Microcapsules

The chosen recipe for producing the microencapsulation system was modified according to our previously reported chitosan encapsulated jojoba oil microcapsules recipe (Cheng *et al.*, 2010). Figure 3.1 shows the flow diagram of chitosan-based microcapsule formation. Zero point five percent, 1%, 1.5% and 2% chitosan solutions were prepared with 1% acetic acid. One percent of NaOH and 1% Triton X-100 were prepared with deionized water. To prepare the microcapsules, 0.05g/mL, 0.1g/mL, 0.15g/mL and 0.2g/mL oil was dispersed in

the chitosan solution respectively with 5 minute stirring using the magnetic stir plate (Heidolph – MR 3001) in order to form an emulsion. The emulsion was then subjected to an ultrasonic processor (Vibra Cell™ VCX 750, Sonics and Materials Inc., USA) with the ultrasonic amplitude of 90% for 15 seconds to break down the emulsion into smaller droplets and avoid the phase separation (Figure 3.2). One percent of NaOH was dropped into the emulsion with different stirring speed, at 600rpm, 800rpm, 1000rpm and 1200rpm until reaching the desired pH values (6, 8, 10 and 12).

The newly developed microcapsules were obtained after centrifugation at 40 rpm for 15 minutes. The microcapsules obtained were rinsed with n-hexane followed by deionized water and then dislodged other unwanted substances using the centrifuge again. The rinsed microcapsules were dispersed with 1% Triton X-100 by stirring them for 3 days.

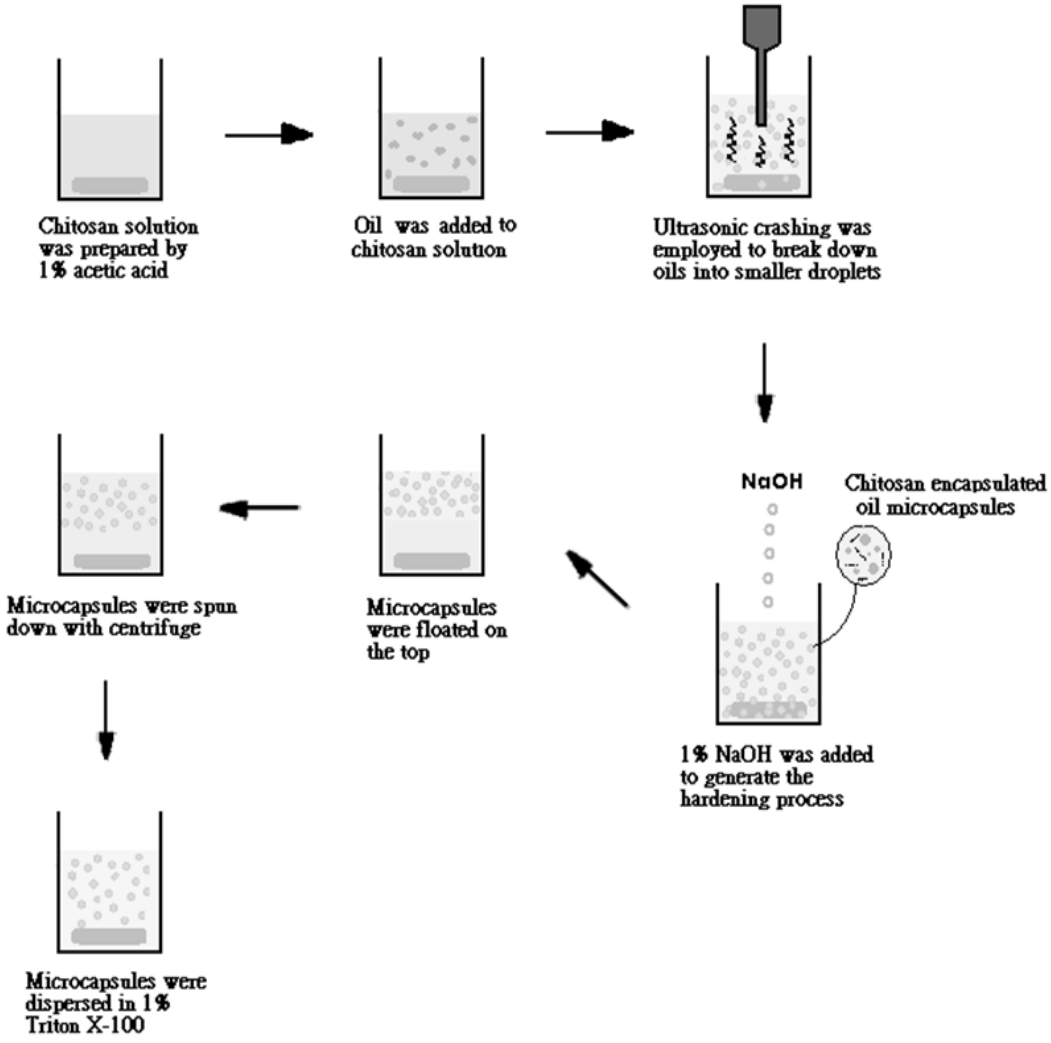


Figure 3.1 Flow diagram of chitosan-based microcapsules preparation

Figure 3.2 Emulsion mixture of chitosan solution and calendula infused oil: before ultrasonic crashing (left); after ultrasonic crashing (right)

3.2.3 Determination of Encapsulation Efficiency

Three measurements of each microcapsule sample were taken. n-hexane was added to the developed microcapsules. The process was repeated for 5 times in order to ensure the complete extraction of free oils. The filtrate was placed on the hot plate at 120℃ to remove the solvent for free oil determination, and the residual free oil was then weighted after cooling. The encapsulation efficiency (EE%) could be calculated by the following formula.

$$EE\% = \frac{\text{Oil contents in microcapsules}}{\text{Total amount of carrier oils}} \times 100\%$$

3.2.4 Determination of Size of Microcapsules

Microcapsules were dispersed in 1mM KCl solution, used as a non-dissolving dispersion medium. The particle size of oil/chitosan microcapsules was determined using a particle size analyzer (MALVERN instruments, ZETASIZER 3000 HSA, United Kingdom).

3.2.5 Morphological Analysis of Microcapsules

Scanning Electron Microscopy (SEM) is a microscopy technique using electron rather than light to produce an image. SEM produces images with three dimensions and high resolution which means precise features can be evaluated at a high magnification. In this case, the morphologies of chitosan microcapsules were examined by a scanning electron microscope (JEOL JSM – 6490, USA). The SEM was operated with 20 KV accelerating voltage at a high magnification up to 4,500X to observe the surface morphology. Few microcapsules were dropped to a silicon wafer and let them dry at room temperature. The test samples were gold coated using a sputter coater – SCD 005 (BAL-TEC,

Liechtenstein) prior to conducting SEM observation.

3.2.6 FTIR Analysis of Microcapsules

Fourier transform infrared spectroscopy (FTIR) is a measuring skill used for examining the chemical bonding and performing the quantitative analysis of minerals by collecting the infrared spectra signals from the test samples. In FTIR analysis, pure ionic minerals such as NaCl or KBr are used because they have no pronounced absorption peaks over the mid-infrared band. In this section, microcapsules were pelleted with KBr through the vacuum process, and the sample/KBr pellet should be transparent when used. Fourier transform infrared spectroscopy analysis was carried out using a Spectrum 100 Series FT-IR Spectrometer (PerkinElmer, USA). In this part of our study, the diffuse reflectance mode measurement was applied in the range of wavelengths from 4000 to 700 cm^{-1} in wave-numbers.

3.2.7 *In Vitro* Controlled Release

The *in vitro* digestion model used was modified from that used by Klinkesorn and McClements (2009). A given amount of developed microcapsules was suspended in a primary release medium for 4 hours, followed by a secondary release medium for 8 hours at 37℃ and stirred at 100rpm using the magnetic stirrer. The primary and secondary release media consisted of equal volumes of HCl solution pH 1.2 containing 10mg/mL pepsin and 0.2mg/mL lipase (artificial gastric juice) and phosphate solution pH 7.4 containing 10mg/mL trypsin and 3mg/mL lipase (artificial intestine juice) respectively. For *in vitro* skin release model of microcapsules, a given amount of developed microcapsules was suspended in a phosphate solution pH 7.4 medium for 120 hours at room

temperature (Pertovaara *et al.*, 1996) and stirred at 100rpm using the magnetic stirrer. Aliquots were taken at desired time intervals, rinsed by n-hexane and centrifuged for 10 minutes at 6000rpm. The fresh medium was then added to the suspension. The supernatant was heated at 120℃ to remove the solvent in order to determine the released oil content. The oil was weighted after cooling. Fresh release medium was added to maintain the total volume of the system's constant.

3.2.8 *In Vitro* and *In Vivo* Toxicity Studies

In order to investigate the availability of the beneficial oil containing chitosan microcapsules for both oral and topical applications, the cytotoxicity studies of chitosan microcapsules were conducted on human skin keratinocytes and C57BL/6 mice. Sulforhodamine B assay was used for cell viability evaluation. Human cells including HaCaT skin keratinocytes were removed from sterile cell culture flasks with trypsin and neutralized with fetal bovine serum. After washing with phosphate buffered saline and centrifugation, skin cells were re-suspended in complete cell culture medium at a concentration of approximately 1×10^5cells/mL and counted manually using a haematocytometer under an inverted microscope. Human cells seeded in the microtitre plates for 24 hours were prepared for the screening of our microcapsules. Microcapsules were stored at a stock concentration of 1g/mL. Microcapsules were added at a starting concentration of 100mg/mL followed by a dilution to 75mg/mL, 50mg/mL and 25mg/mL respectively and incubated with cells for a further period of 48 hours. Doxorubicin was used as a positive control. Afterwards, the evaluation of possible toxic potential was performed by the sulforhodamine B protein staining methods. Briefly, skin cells were fixed with trichloroacetic acid, washed with distilled water and stained with sulforhodamine B. Afterwards, cells were

washed again with acetic acid and stained cells were dissolved in unbuffered Tris-base. Finally, optical absorptions were measured at 575nm using a microplate reader (Victor V form Perkin Elmer, Life Sciences).

Eight weeks old C57BL/6 mice, weighing approximately 20-25g, were purchased from the animal unit of The Chinese University of Hong Kong and maintained in a conventional sanitary facility, in accordance with the institutional guidelines on animal care, with the required consistent temperature and relative humidity. All the procedures were approved by the Animal Research Ethics Committee. A total of 8 mice were included in our preliminary study and they were divided into two groups. The chitosan microcapsules were mixed with the physiological saline solution. From day one to day three, treatment groups received chitosan microcapsules once daily at a concentration of 20mg/g body weight (or 2mg oil/g body weight). Control group received the same volume of physiological saline orally. The animals were monitored and any change in the body weight was recorded. On day four, all the mice were sacrificed and autopsies were performed to collect peripheral blood and livers for analytical chemistry and pathological analysis. Sections of mouse liver from autopsy samples were dewaxed with xylene and gradient concentrations of ethanol. Slides were stained with haematoxylin and finally they were inspected under a light microscope for any possible necrotic features. Whole blood was collected after the mice were sacrificed and plasma was isolated after centrifugation. Afterwards, plasma liver enzymes including alanine aminotransferase (ALT) and asparate aminotransferase (AST) were measured by the Vet biochemistry assay kits for the IDEXX laboratories machine to determine whether there are any liver failure phenomena from chitosan microcapsules treated groups of mice by

comparing with the vehicle treatment group and the normal control ranges. (Chui *et al.*, 2009; Wang *et al.*, 2010).

3.2.9 Application of Calendula Oil Loaded Chitosan Microcapsules on Textile Materials

One hundred percent cotton fabric samples were cut into many pieces of 20cm × 20cm. Chitosan microcapsules containing Calendula oil were firstly diluted with deionized water at the liquor ratio of 1:10. The solution was stirred for 15 minutes using a magnetic stir plate at a speed of 1200rpm to obtain a better dispersion of microcapsules. A binder (Aldrich, Germany) was used to enhance the fixation of chitosan microcapsules onto the cotton fabric. The microcapsule solution was sprayed on the fabric sample followed by drying it at room temperature.

3.3 Results and Discussion

3.3.1 Influence of Chitosan Concentration and Types of Carrier Oils

The encapsulation efficiency and the particle size can be affected by the wall shell concentrations and types of oil phase of microcapsules. In order to successfully entrap the core substance into the wall shell of microcapsules, the determination of a proper concentration of the wall shell and the type of oil is crucial. Figure 3.3 shows the encapsulation efficiency (EE%) and particle size of microcapsules prepared by using four different chitosan concentrations with three different oils respectively. The results obtained clearly show that the encapsulation efficiency raised with the increment of the chitosan concentration from 0.5%w/v to 1.5%w/v. This was probably due to the fact that chitosan

concentrations are higher than 0.5%w/v which promoted better shell forming ability to entrap the core oils into the microcapsules. On the other hand, it was observed that a slight but reproducible reduction in encapsulation efficiency was found when the chitosan concentration was increased to 2%w/v as too high concentration of chitosan solution leaded to higher viscosity of emulsion in the microencapsulation system, and thereby retarding the formation of microcapsules.

Figure 3.4 shows the particle size of microcapsules prepared by different oils. The mean particle size of microcapsules was ranging from 2.60 to 4.75μm. The average particle size raised with higher chitosan concentration as higher concentration of chitosan solution might be capable of forming a thicker and stronger wall shell of the microcapsule for preventing core oils from rupture (Higuera-Ciapara *et al.*, 2004) and results in larger particle size. The larger particle size of microcapsules might also be caused by the higher chitosan concentration because of its high gel forming ability or selfaggregation at alkaline pH during the microcapsule formation process. The encapsulation efficiency was the highest when the chitosan concentration was 1.5%w/v while the mean particle size was the smallest when chitosan was used at 0.5%w/v. It was also observed that microcapsules prepared with calendula oil showed the highest encapsulation efficiency when compared to other two oils, revealing that this oil had higher affinity to the chitosan-based microencapsulation system. Following the reported results, the chitosan concentration (1.5%w/v) and the calendula oil were selected to prepare microcapsules for the remaining studies.

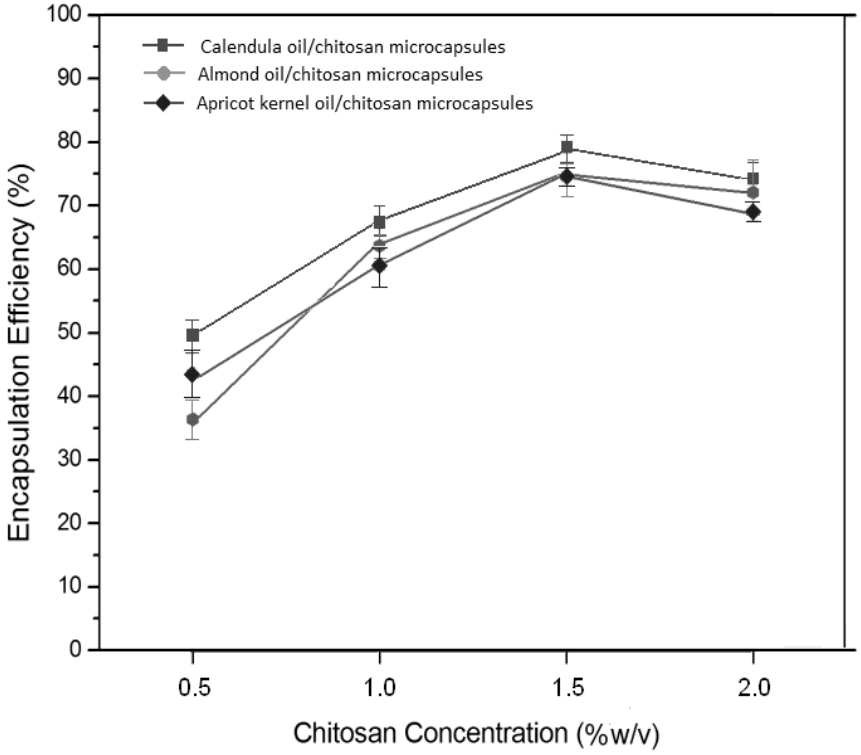


Figure 3.3 Influence of chitosan concentration on encapsulation efficiency

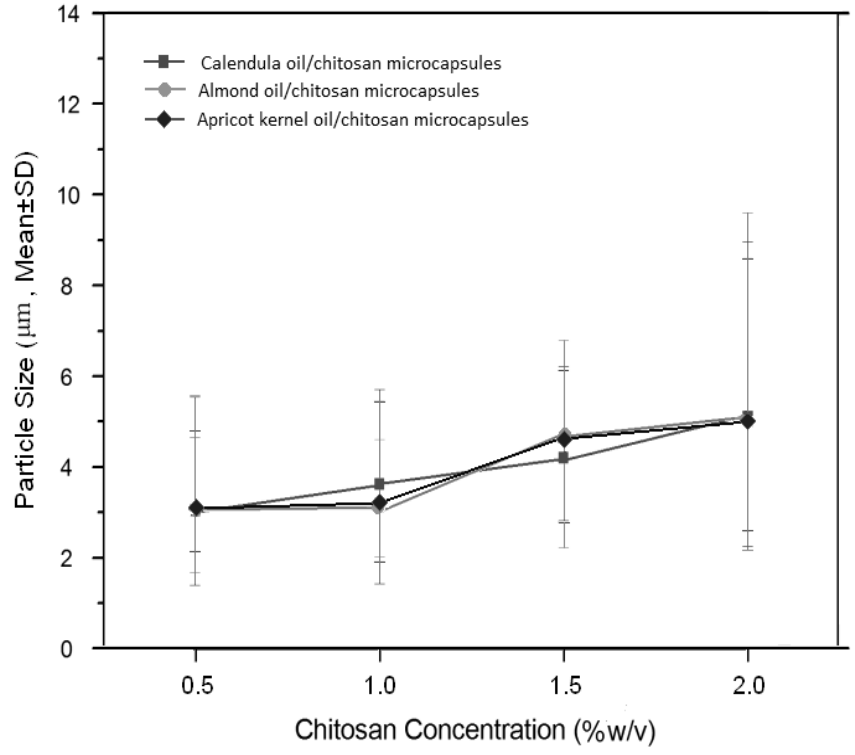


Figure 3.4 Influence of chitosan concentration on particle size

3.3.2 Influence of Core/Wall Ratio

The encapsulation efficiency and the particle size of microcapsules were not only influenced by the wall shell concentration, but also by the core/wall ratio. As shown in Figure 3.5, the mean particle size slightly increased while the encapsulation efficiency decreased when the core/wall ratio increased from 0.05 g/ml to 0.2 g/ml. There was no observable significant difference in particle size variation. By comparing two indexes, it could be concluded that the best quality of microcapsules can be obtained by selecting 0.1 g/ml of core/wall ratio.

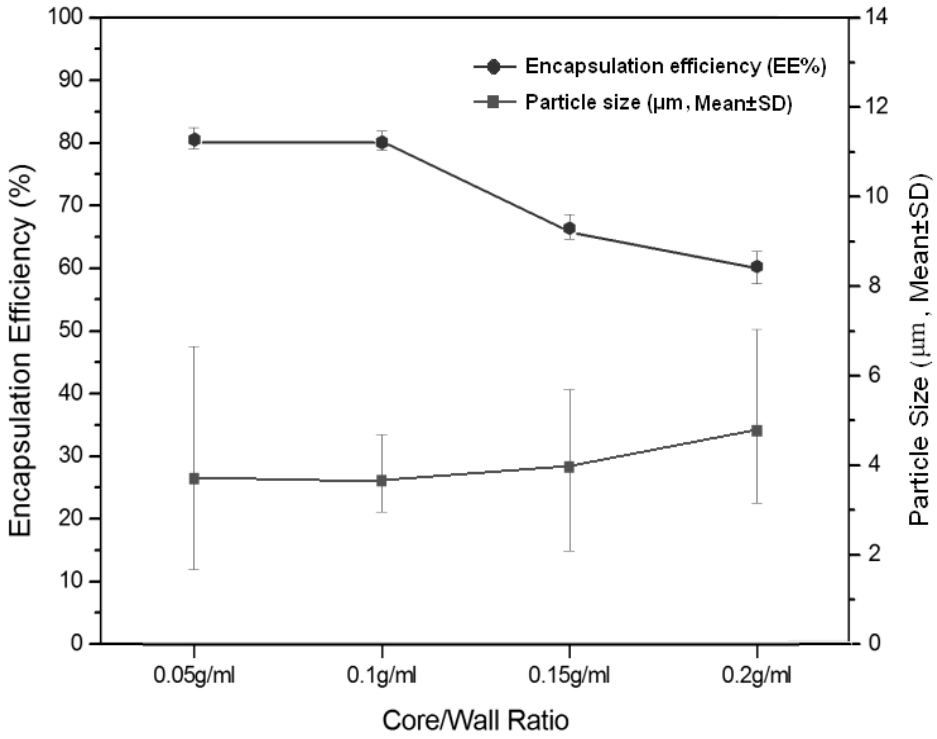


Figure 3.5 Influence of core/wall ratio on encapsulation efficiency and particle size

3.3.3 Influence of Stirring Speed

Figure 3.6 shows the encapsulation efficiency and the particle size of microcapsules obtained by using different stirring speed. It was observed that, as

the stirring speed increased, the encapsulation efficiency increased while the mean particle size decreased. This was because increasing the emulsion stirring speed was able to produce microcapsules with smaller particle size and prevent the microcapsules from aggregation during the microcapsule generation process (Hsieh *et al.*, 2006). As the stirring speed decreased, larger clumping occurred during the hardening process, retarding the formation of microcapsules. As a result, lower encapsulation efficiency and larger mean particle size were obtained at lower stirring speed. In order to produce microcapsules with better quality, the stirring speed of 1200 rpm was considered to be the optimal condition for further studies.

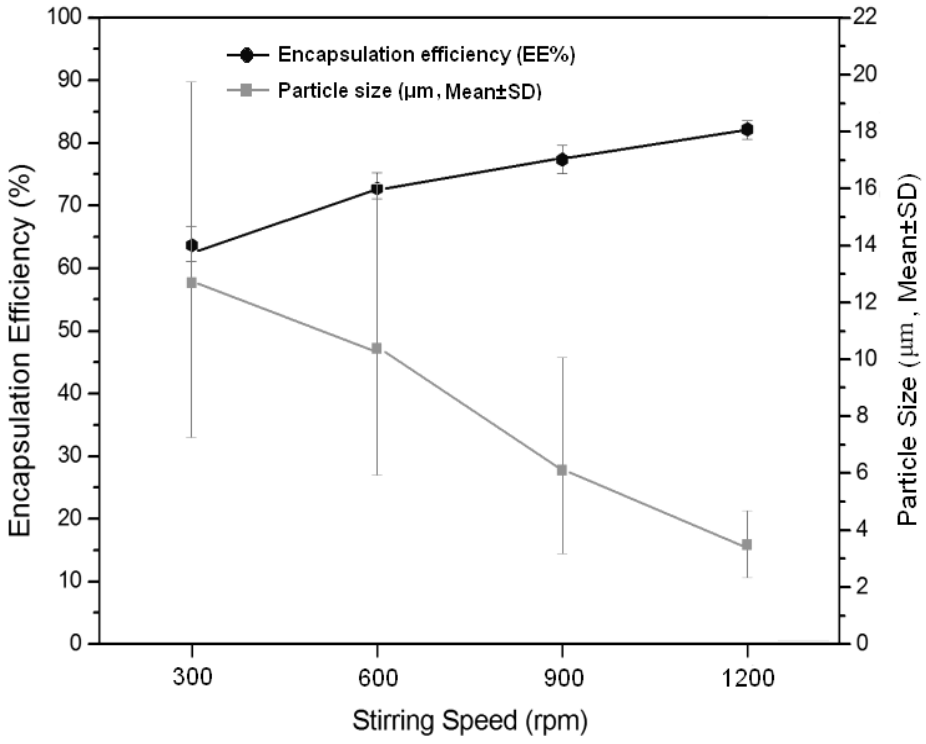


Figure 3.6 Influence of stirring speed on encapsulation efficiency and particle size

3.3.4 Influence of pH Value

Figure 3.7 shows the encapsulation efficiency and the particle size of microcapsules at different pH value. It was noted that the encapsulation efficiency increased with an increment in pH value from 6 to 10 and reached its maximum level. Conversely, the mean particle size decreased with the increase of pH value from 6 to 10 and then slightly increased. This could be explained by insufficient hardening of microcapsules. Inadequate input of hardening agent (NaOH) may not produce the thick and stable wall shell of microcapsules leading to the higher tendency of core oil leakages, and eventually, lower encapsulation efficiency. A higher extent of aggregation may be present in microcapsules with weaker wall shell and more oil leakages, resulting in larger mean particle size (Hsieh *et al.*, 2006). It was confirmed that an appropriate amount of the hardening agent was essential for developing the microcapsules with higher encapsulation efficiency (lesser oil leakages) and smaller particle size (low extent of clumping between microcapsules). Therefore, pH 10 was selected to produce microcapsules with the best quality.

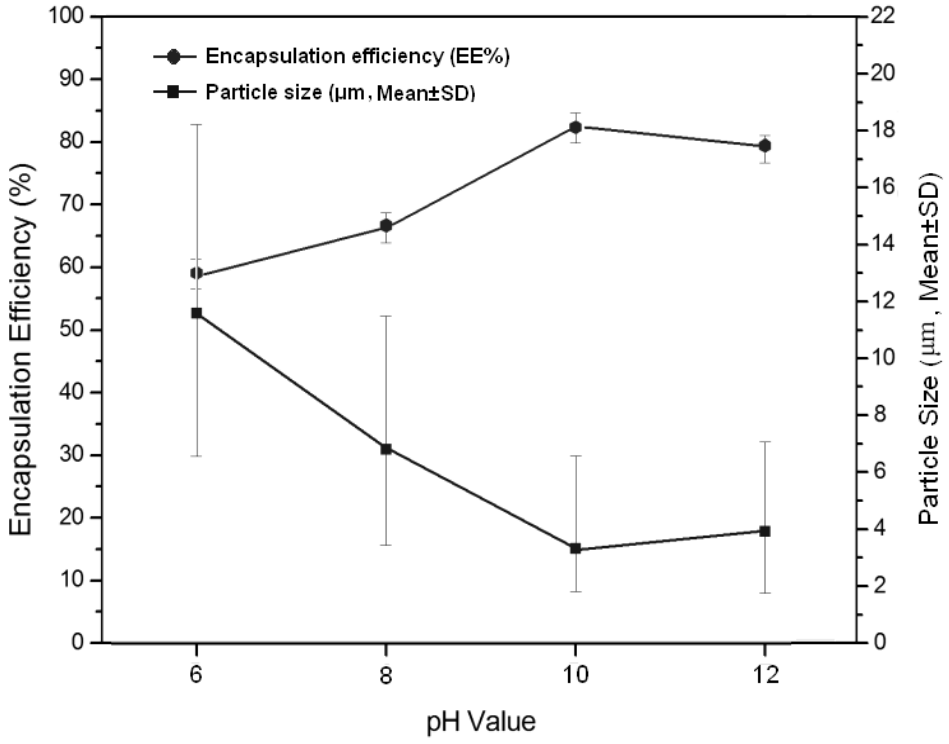


Figure 3.7 Influence of pH value on encapsulation efficiency and particle size

3.3.5 Surface Morphology Analysis

The SEM image of calendula infused oil/chitosan microcapsules prepared with the optimal parameter condition with the magnification at 4500x is shown in Figure 3.8. It is evident that the microcapsules were approximately spherical in shape and smooth in surface morphology without aggregation. The microcapsules were ranging from 1.71 to 6.79μm with a mean particle size of 3.78μm.

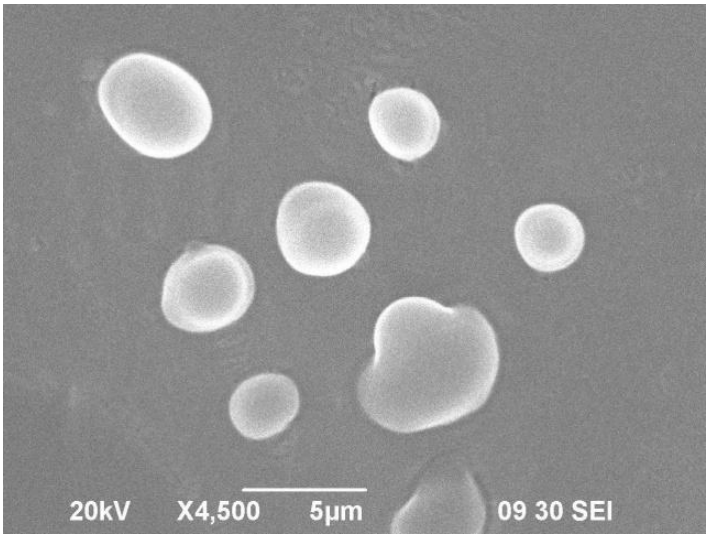


Figure 3.8 SEM image of calendula infused oil/chitosan microcapsules (Chitosan concentration=1.5%; core/wall ratio=0.1g/mL; stirring speed=1200rpm and pH value=10)

3.3.6 FTIR Spectrum Analysis

The infrared absorption spectra of chitosan, calendula infused oil and calendula oil/chitosan microcapsules are shown in Figure 3.9. Significant peaks were observed in chitosan spectrum at 3445cm^{-1} and 1649cm^{-1} that were related to the O-H group hydrogen bond) and the C=O stretching (amide I) respectively. On the other hand, the Calendula oil had the significant peaks at 2853cm^{-1} and 2923cm^{-1}, that were related to the C-H aliphatic stretching, while the peak at 1744cm^{-1} was referred to the C=O stretching (carboxylic acid ester). The peaks at 1464cm^{-1} and 1160cm^{-1} were also representing the C=C stretching and C-O stretching (ester) respectively.

The calendula oil/chitosan spectra showed the specific peaks of chitosan and calendula oil. The absorbance of O-H group at λ = 3449cm^{-1} and C=O stretching

at $\lambda = 1643cm^{-1}$ showed the existence of chitosan as a wall material of the newly developed microcapsules. The peaks of calendula oil were also observed in the newly developed microcapsule spectra. The absorbance of calendula oil from the band of C-H aliphatic stretching at $\lambda = 2854cm^{-1}$ and $\lambda = 2927cm^{-1}$, the C=O stretching (carboxylic acid ester) at $\lambda = 1748cm^{-1}$, the C=C stretching at $\lambda = 1464cm^{-1}$ to the band of the C-O stretching (ester) at $\lambda = 1160cm^{-1}$ were all contained in the spectra of calendula oil/chitosan microcapsules. Based on the FTIR results, it was observed that the newly developed microcapsules contained the chemical ingredients and functional groups of the wall material (chitosan) and the core active ingredient (calendula oil). The overall results confirm the synthesis of the newly developed microcapsules.

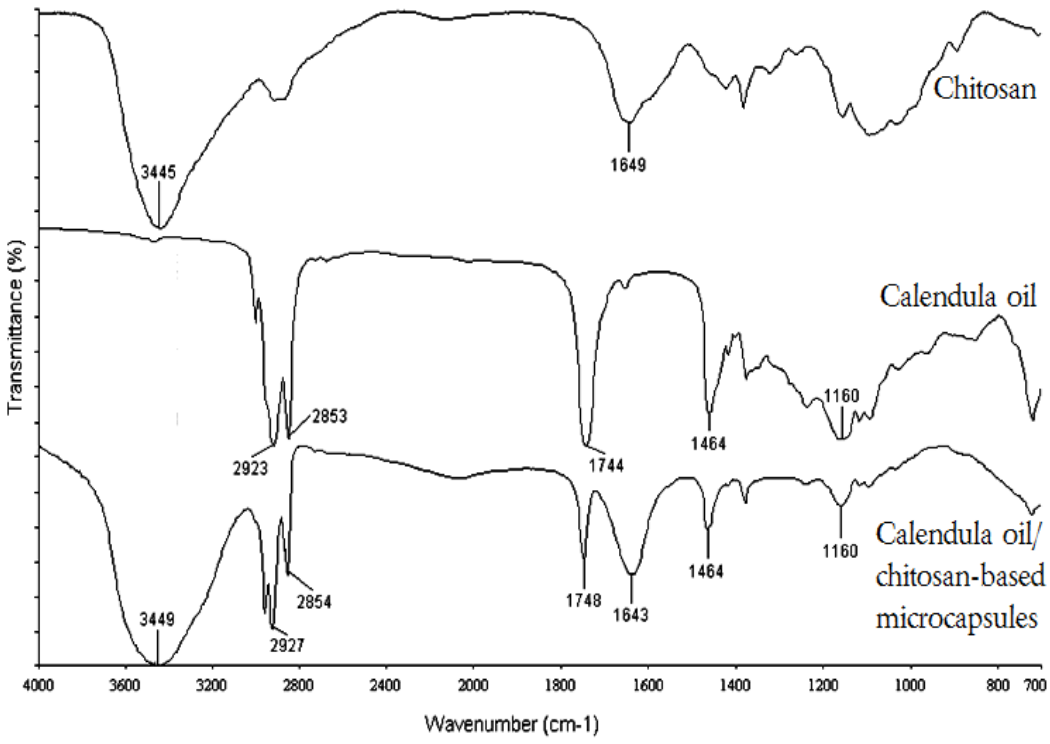


Figure 3.9 FTIR spectrum of calendula oil/chitosan microcapsule

3.3.7 *In Vitro* Controlled Release of Microcapsules

The percentage of oil release was monitored for 12 hours and shown in Figure 3.10. The oil was more readily released from the chitosan-based microcapsules when subjected to the primary release medium of HCl (pH 1.2). It was observed that approximately half of the oil was released during the first 4 hours in HCl medium mimicking the stomach condition in human body. This may be associated with the fact that the chitosan wall shell of microcapsules was more susceptible to the acidic condition, as chitosan was found to be soluble in acidic medium, resulting in higher leakages of core oils (Peniche *et al.*, 2004; Klinkesorn and McClements, 2009). The oils were released at a slower speed in the secondary medium of phosphate (pH 7) as chitosan was more stable and became more rigid in alkaline condition. Approximately 75% of the oil was released after 12 hours. The calendula oil was released at a slower speed in the secondary medium of phosphate (pH 7); approximately 75% of the oil was released after 12 hours.

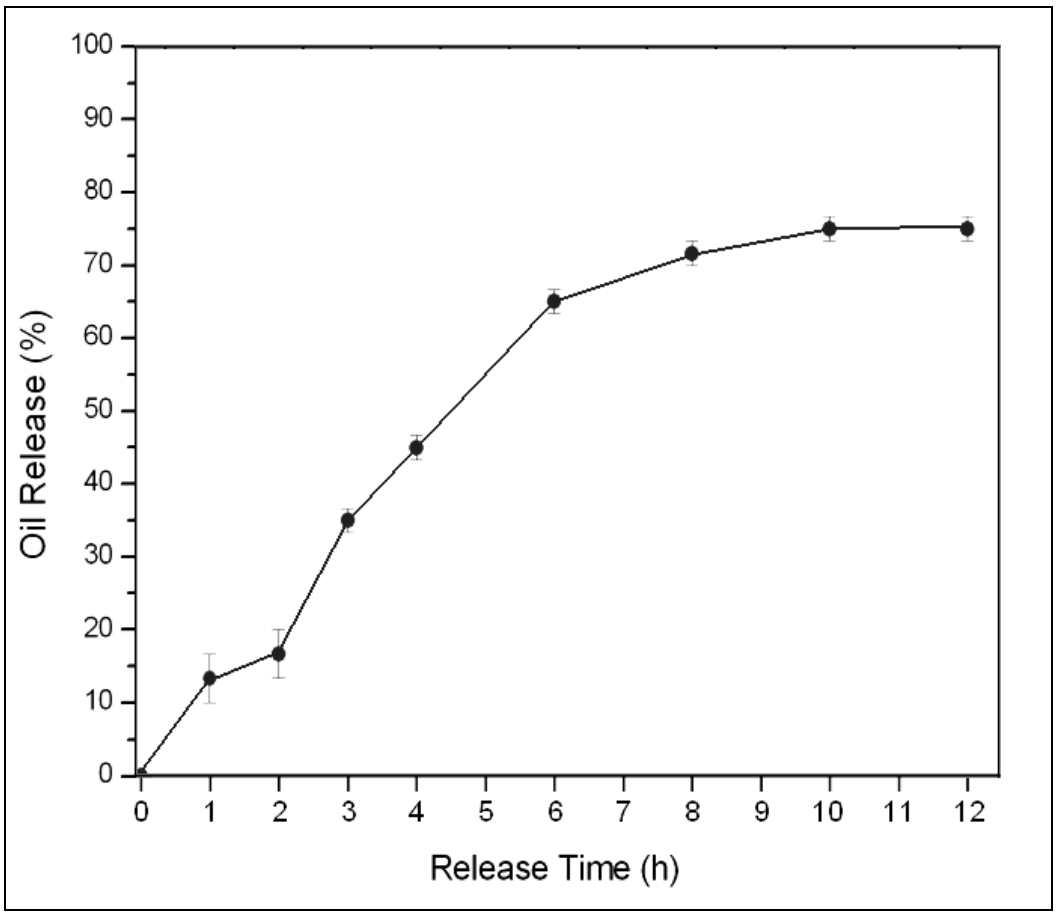


Figure 3.10 Release profile under simulated digestion system of calendula oil/ chitosan microcapsule

In the *in vitro* skin release model which simulated the skin condition, it was observed that calendula infused oil was gradually released from the chitosan wall of microcapsules. After 120 hours, there was approximately 80% of the oil released from microcapsules, as shown in Figure 3.11.

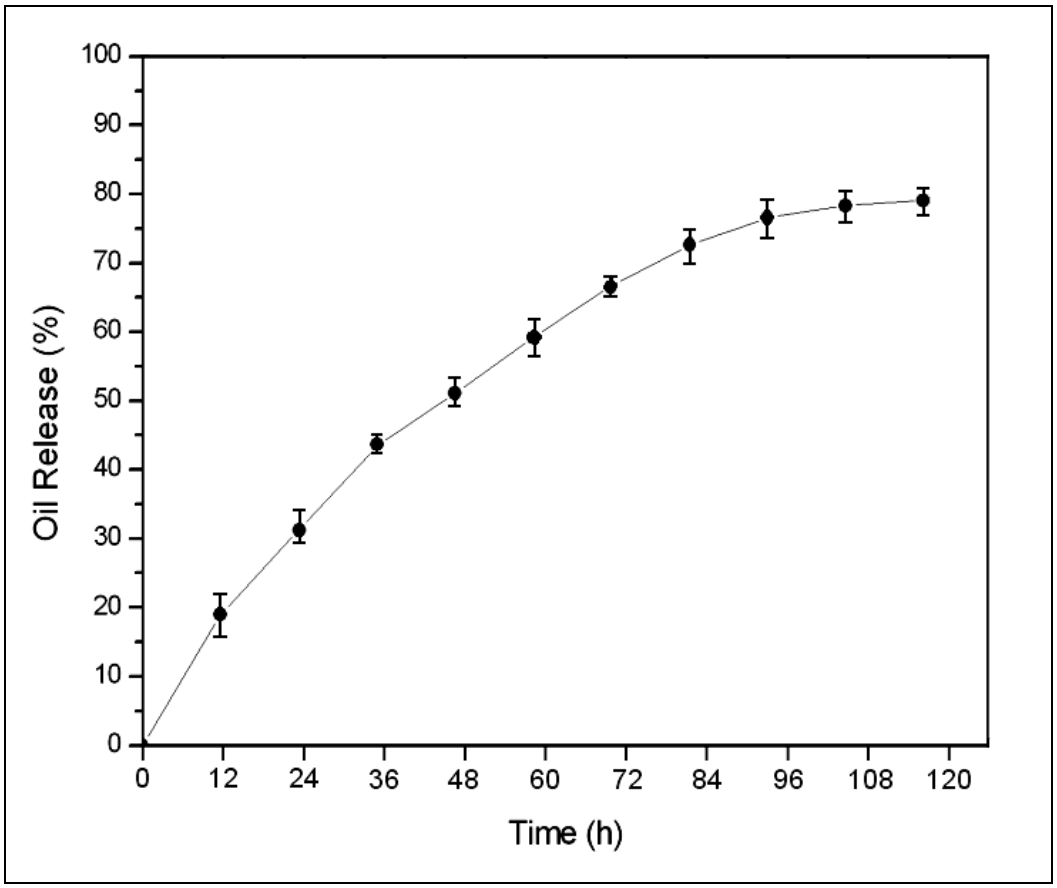


Figure 3.11 Release profile under simulated topical condition of calendula oil/ chitosan microcapsule

3.3.8 *In Vitro* and *In Vivo* Toxicity Studies

In order to investigate whether the developed microcapsules are expected to be safe when administered topically and orally, both *in vitro* and *in vivo* tests were conducted. We first demonstrated that the developed microcapsules exert no cytotoxic effects on human skin cells at a maximum concentration of 100mg/mL after 48 hours of incubation. As shown in Figure 3.12, the developed microcapsules, chitosan and calendula oil exhibited satisfactory cell viability over the entire concentration range and more than 90% viability was present even when the concentration was up to 100mg/mL. There was no observable cellular damage after the treatment of 100mg/mL microcapsules (Figure 3.13A and

3.13B). The chitosan microcapsules containing calendula oil were therefore proven to be non-cytotoxic towards the human HaCaT cells.

Since liver is the front line detoxification organ for any possible toxic substance, we aimed to investigate whether the hepatocytes will be affected after the ingestion of microcapsules. The *in vivo* toxicology analysis on mice further proved that when the microcapsules were administrated orally at 20mg of microcapsules per gram of body weight of mice or 2mg of oil per gram of body weight did not exert any significant damage including necrotic feature to the liver. Figure 3.13D shows a representative mouse with normal morphology of hepatocytes after taking developed microcapsules (the control is shown in Figure 3.13C) suggesting that there are damages of the liver cells under these experimental conditions. This conclusion was further sustained by analysis of the plasma liver enzyme levels, demonstrating no statistical significant difference is found between the microcapsule group in respect to the vehicle group ($p>0.05$) (Figure 3.13E). Assuming that an adult has a body weight of 50kg, the results here speculated that a person could take an average of up to 1kg microcapsules or 100g oil daily without adverse toxic effect. Additionally, all microcapsule-treated mice still survived and no observable change in their body weight occurred after the treatment.

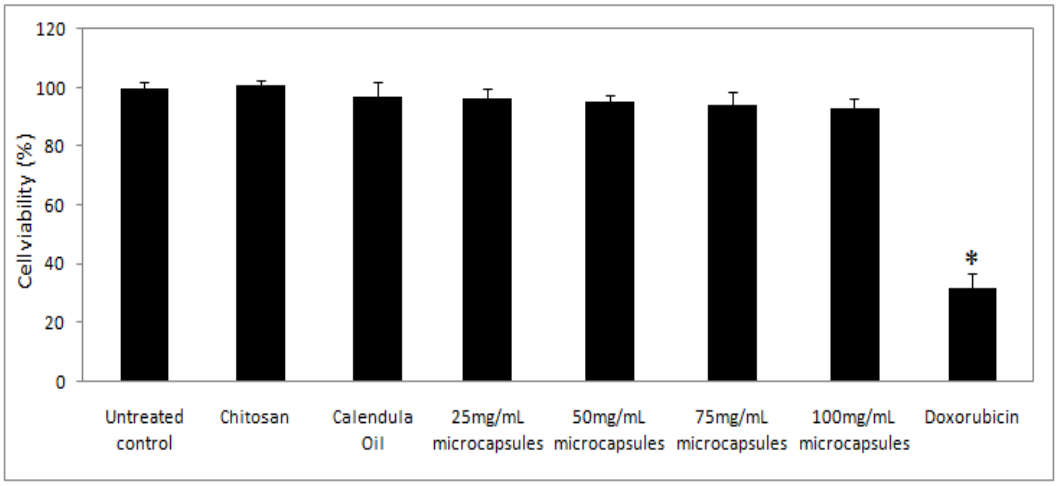
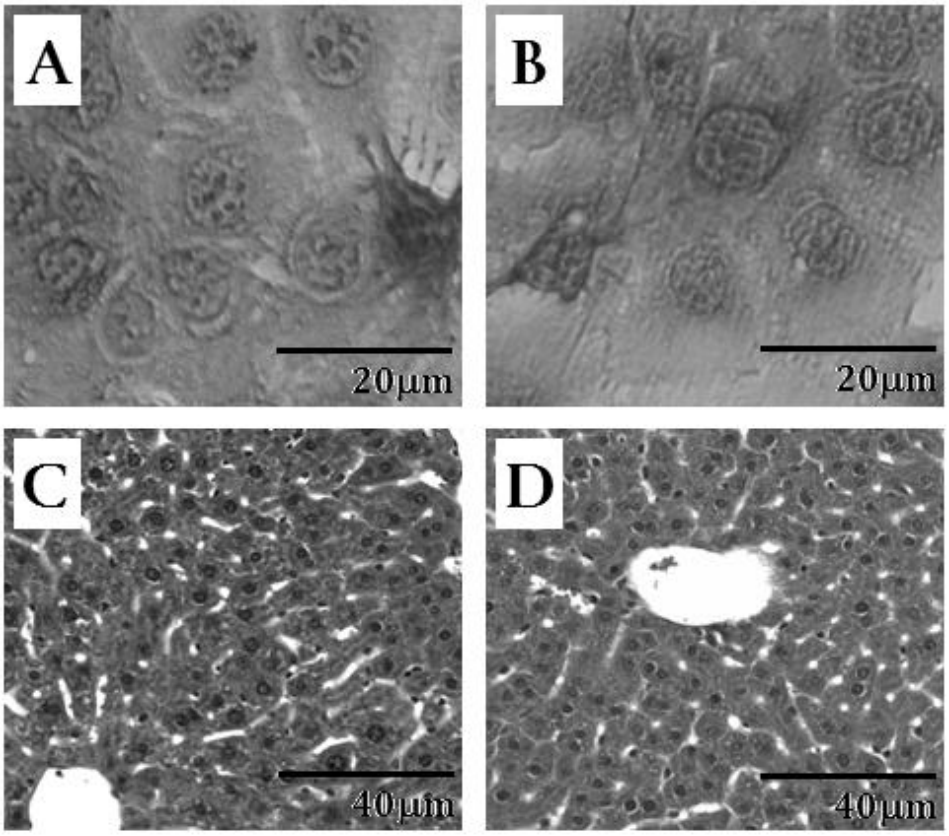


Figure 3.12 Cytotoxicity evaluation of microcapsules on human skin keratinocytes (*p<0.05)



E	ALT(U/L)	AST(U/L)
Vehicle group	53.7±13.9	62.3±33.5
Microcapsule group	61.1±14.1	61.2±27.4
Reference value	28-132	59-247

Figure 3.13 (A) Vehicle treated control human skin keratinocytes; (B) 100mg/mL microcapsules treated human skin keratinocytes; (C) Vehicle treated control mouse; (D) Microcapsules treated mouse (20 mg of microcapsules per gram of body weight of mice OR 2 mg of oil per gram of body weight of mice); (E) Analysis of the plasma liver enzyme levels. Results are shown as mean ± standard deviations from each group. No statistical significant difference is found between the microcapsule group in respect to the vehicle group (p>0.05)

3.3.9 Application of Calendula Oil/ Chitosan Microcapsules on Textile Materials

Figure 3.14 and 3.15 show the SEM image of cotton fibres exhibiting the fibrous structure and cotton fibres treated with chitosan microcapsules respectively. The SEM results showed that microcapsules were fabricated on the fibre surface during the spraying process. The core beneficial oil of microcapsule-treated textiles were believed to be gradually released under friction and pressure during human wear, people could then experience the health advantages via a simple daily wear.

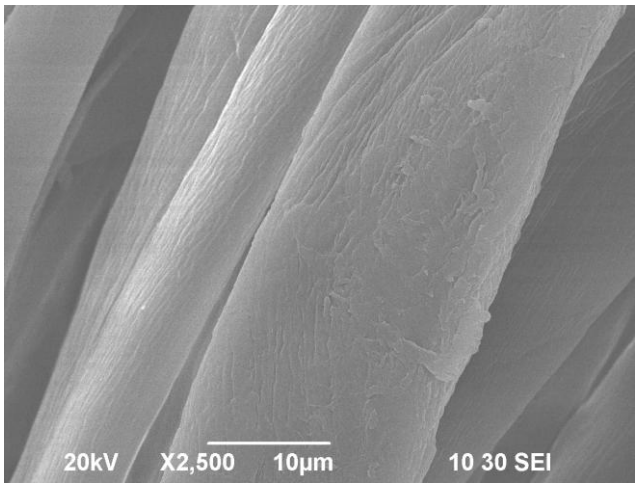


Figure 3.14 SEM image of control cotton fibres

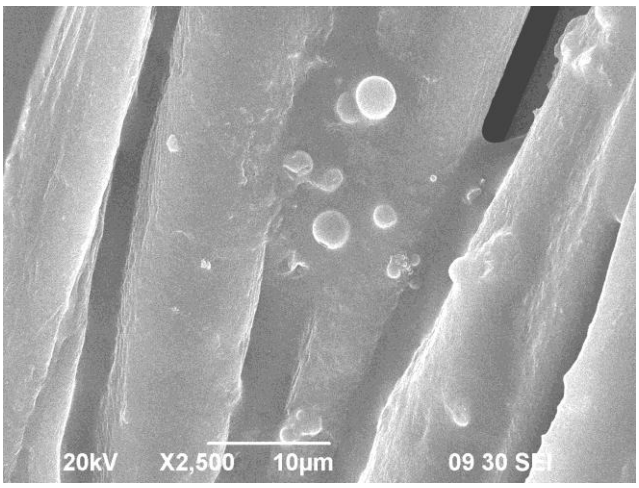


Figure 3.15 SEM image of cotton fibres treated with chitosan microcapsules

3.4 Conclusion

Chitosan-based microcapsules were prepared with chitosan as a wall shell material and calendula oil as the core substance using the simple coacervation technique. By analyzing the encapsulation efficiency and mean particle size, the optimal parameter combinations were achieved as follows: concentration of chitosan: 1.5%w/v; core/wall ratio: 0.1g/ml; stirring speed: 1200rpm; pH value: 10. By using these optimal conditions, the encapsulation efficiency and the mean particle size were 82.37% and 3.78 μm respectively. The SEM image showed that the developed microcapsules were relatively in spherical form with minimum aggregation. The FTIR spectrum also demonstrated the encapsulation of calendula oil into chitosan wall shell of microcapsules. It was observed that approximately 75% of the oil was released after 12 hours in the simulated gastro-intestinal condition while approximately 80% of the oil was released from microcapsules after 120 hours in the skin release model.

The *in vitro* cytotoxicity evaluation demonstrated that the microcapsules exhibited no significant cytotoxic effects on human keratinocyte skin cells at a maximum concentration of 100mg/mL. The calendula oil containing chitosan microcapsules were therefore shown to be non-cytotoxic towards the human HaCaT cells. The *in vivo* toxicology analysis on mice further demonstrated that when the microcapsules were administrated orally at 20mg of microcapsules per gram of body weight of mice or 2mg of oil per gram of body weight did not show any significant damage to the liver section. Both toxicity results suggested that the calendula oil/ chitosan microcapsules could be applied both orally and topically without any adverse cytotoxic effects. The microcapsules were also fabricated onto cotton fabrics and the released calendula oil from microcapsules

was believed to promote the health benefits in our daily wear. Further experimental work for elucidating the possibility and safety uses of the calendula oil/chitosan microencapsulation as a drug carrier both topically and orally will be mentioned in the following chapters.

CHAPTER 4

DEVELOPMENT OF HYDROCORTISONE SUCCINIC ACID/ CHITOSAN MICROCAPSULES FOR ORAL DRUG DELIVERY

4.1 Introduction

Microencapsulation technology has a high potential for biomedical applications by imparting conventional drug dosages to the human body in a more effective route. As many drugs are present the problems of low bioavailability when they are administrated through oral routes. Introducing microencapsulation technology to oral drug delivery system is believed to minimize the problems in the conventional drug administration, also promoting an alternative administration route to improve the release profile and bioavailability of the drug dosage. In accordance with the results generated before, the optimal parameter combination was selected to develop the chitosan-based microcapsules for entrapping hydrocortisone succinic acid (HSA) so as to propose another administration routes for oral drug delivery.

Hydrocortisone (HS) is a synthetic form of corticosteroid administrated when the body is deficient in the natural hormone. The major clinical usages of HS include (Wang *et al.*, 2009): (1) anti-inflammation which can reduce and prevent tissue inflammation; (2) action on immune-system which can reduce and prevent the cell mediated immune-responses as well as allergy; (3) anti-virus and anti-coma which can reduce the body response towards viral infections, reduce cellular damages and protect the human body. In fact, many drugs are chemically modified to give more water soluble derivatives and their clinical responses are determined by the conversion into their parental drugs. Hydrocortisone sodium

succinate (HSS) is in the form of hydrocortisone ester sodium salt. It is practically more soluble in aqueous solution than HS. Therefore it is commonly used in aseptic formulations (Rigge and Jones, 2005). The side effect of HS has been proven to be reduced when using the insulin/alginate shell as a carrier for drug delivery (Zhao *et al.*, 2011). HS can be used in oral administration, topical application and intravenous injection. It generally possesses low bioavailability when they are orally administrated and topically applied while some patients are found to have severe allergic response towards intravenous injection.

The safety use of calendula oil/ chitosan microcapsules as a possible carrier for oral delivery has been proven in Chapter 3. This chapter explains how to further make use of the microencapsulation technology to encapsulate the hydrocortisone succinic acid (HSA) into chitosan wall shell in order to investigate their pharmacological activity using the plasma adrenocorticotropic hormone (ACTH) of C57BL/6 mice.

4.2 Experimental

4.2.1 Materials

Chitosan (75%-85% deacetylated, from Aldrich, Germany) was dissolved in 1% acetic acid. Calendula infused oil was purchased from Easy Creation Asia Limited, Hong Kong. Hydrocortisone succinate was obtained from Pharmica, Switzerland. All reagents were purchased from Sigma-Aldrich, Germany.

4.2.2 Preparation of Hydrocortisone succinic acid/ Chitosan Microcapsules

Hydrocortisone succinate was converted to its conjugated acid (HSA) after

acidification and purification. Twenty milligrams of HSA was firstly dissolved in 2g of calendula infused oil and the synthesis processes were then performed as described in Chapter 3, Section 3.2.2.

4.2.3 Determination of Drug Loading Efficiency

Drug loaded microcapsules were dissolved in 0.1N HCl. The samples were then filtered. HSA content was analyzed by measuring the absorbance at 270nm (λ_{max} of drugs in 0.1N HCl) after suitable dilution using UV VIS spectrophotometer (Lambda 18, Perkin Elmer, USA). Each sample was performed in triplicate in order to obtain the mean drug loading efficiency. The drug loading efficiency was calculated as below. Figure 4.1 shows the standard calibration curve of HSA.

$$\text{Drug loading efficiency (\%)} = \frac{\text{Calculated drug concentration}}{\text{Theoretical drug concentration}} \text{ X } 100$$

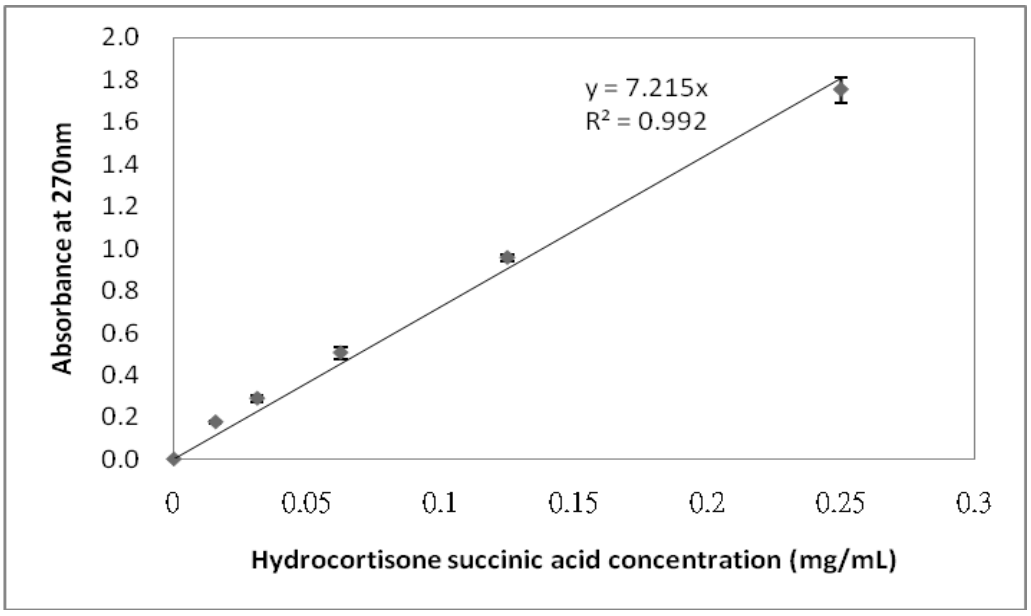


Figure 4.1 Standard calibration curve of HSA

4.2.4 Size of HSA/Chitosan Microcapsules

The particle size of drug-loaded microcapsules was investigated as described in Chapter 3, Section 3.2.4.

4.2.5 Morphological Analysis of HSA/Chitosan Microcapsules

Surface Morphology of drug-loaded microcapsules was evaluated as described in Chapter 3, Section 3.2.5.

4.2.6 FTIR Analysis of HSA/Chitosan Microcapsules

FTIR spectrum of drug-loaded microcapsules was analyzed as described in Chapter 3, Section 3.2.6.

4.2.7 *In Vitro* Controlled Release of HSA/Chitosan Microcapsules

The *in vitro* release model of HSA/chitosan microcapsules was modified from the recipe used by Klinkesorn and McClements (2009). A given amount of developed microcapsules was suspended in a primary release medium for 4 hours, followed by a secondary release medium for 8 hours at 37℃ and stirred at 100rpm. The primary and secondary release media were composed of equal volumes of HCl solution pH 1.2 containing 10mg/mL pepsin and 0.2mg/mL lipase (artificial gastric juice) and phosphate solution pH 7.4 containing 10mg/mL trypsin and 3mg/mL lipase (artificial intestine juice) respectively. Aliquots were taken at desired time intervals. The drawn sample was filtered using filter paper and the residue was then returned to the suspension medium. The clear filtrate was analyzed at λ_{max}= 270nm by UV VIS spectrophotometer (Lambda 18, Perkin Elmer, USA) for the determination of drug content. Each sample was conducted in triplicate.

4.2.8 Effect of HSA/Chitosan Microcapsules on the Plasma Adrenocorticotropic Hormone (ACTH) of C57BL/6 Mice Model

Eight weeks old C57BL/6 mice, weighing approximately 20-25g, were purchased from the animal unit of The Chinese University of Hong Kong and maintained in a conventional sanitary facility, in accordance with the institutional guidelines on animal care, with the required consistent temperature and relative humidity. All the procedures were approved by the Animal Research Ethics Committee. A total of 9 mice were included in our preliminary study and they were divided into 3 groups respectively. The chitosan microcapsules were mixed with the sterilized water. From day one to day three, treatment groups received chitosan microcapsules which were adjusted to a drug concentration of 10μg/g body weight once daily. Control group received the same volume of sterilized water orally. On day four, all the 9 mice were sacrificed. All their blood was collected and plasma was isolated after centrifugation. The determination of plasma ACTH concentration was based on the instruction from the LumELISA Kit (MYBioSource, USA) provided.

4.3 Results and Discussion

4.3.1 Drug Loading and Particle Size

The mean drug loading efficiency and drug loading of HSA/chitosan microcapsules were examined to be 53.90% ± 1.94 and 1540.02μg/mL ± 55.56 respectively. The particle size of HSA/chitosan microcapsules was ranging from 1.90 to 5.80μm with the mean of 3.56μm.

4.3.2 Surface Morphology

The SEM image of HSA/chitosan microcapsules was shown in Figure 4.2. It was observed that the developed microcapsules were approximately spherical in shape with the relatively smooth surface morphology.

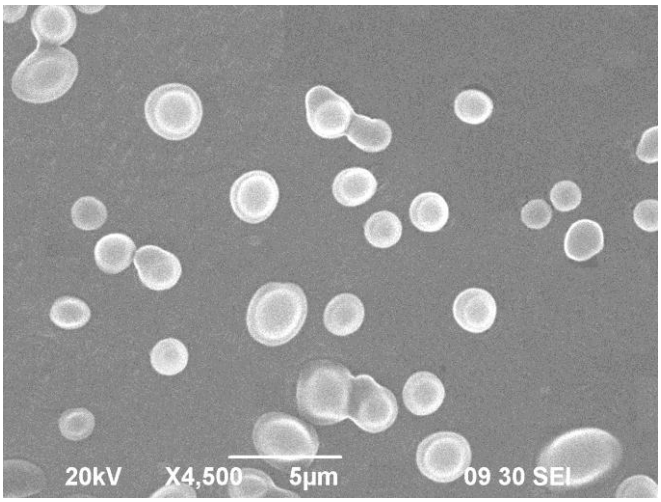


Figure 4.2 SEM image of HSA/chitosan microcapsules

4.3.3 FTIR Spectrum

In this study, the diffuse reflectance mode measurement was applied in the range of wavelengths from 4000 to 600cm^{-1} in wave-numbers. The FTIR spectra of chitosan, calendula infused oil, HSA and HSA/chitosan microcapsules are shown in Figure 4.3. Significant peaks were observed in Chitosan spectrum at 3435cm^{-1} and 1650cm^{-1} which were related to hydrogen-bonded O-H stretching and C=O stretching (amide I) respectively. Calendula infused oil had the significant peaks at 2853cm^{-1} and 2923cm^{-1} (C-H aliphatic stretching), 1744cm^{-1} (C=O stretching) and 1464cm^{-1} (C=C stretching). HSA contained the significant peaks at 3465cm^{-1} (O-H stretching), 1718cm^{-1} (C=O stretching) and 1628cm^{-1} (C=C stretching).

The spectrum of HSA/chitosan microcapsules showed the characteristic peaks of

HSA, chitosan and calendula infused oil. The absorbance at 3437cm^{-1} showed the existence of the O-H stretching from chitosan. The peak at 1630cm^{-1} also detected the presence of C=O stretching from chitosan and HSA of the developed microcapsules. They superimposed with each other. The absorbance at λ = 1718cm^{-1} occurred in microcapsule spectrum showed the C=O stretching from HSA. The absorption of calendula infused oil from the band of C-H aliphatic stretching at λ = 2854cm^{-1} and λ = 2920cm^{-1} existed in the spectrum of the developed microcapsules. The band of the C=O stretching (carboxylic acid ester) at 1744cm^{-1} and the C=C stretching at 1464cm^{-1} revealed the presence of calendula infused oil in drug loaded microcapsules. Based on the FTIR results, the newly developed microcapsules contained the chemical functional groups of the wall material (chitosan) and the core ingredient (HSA dissolved in calendula infused oil). The overall results confirm the synthesis of HSA loaded chitosan microcapsules.

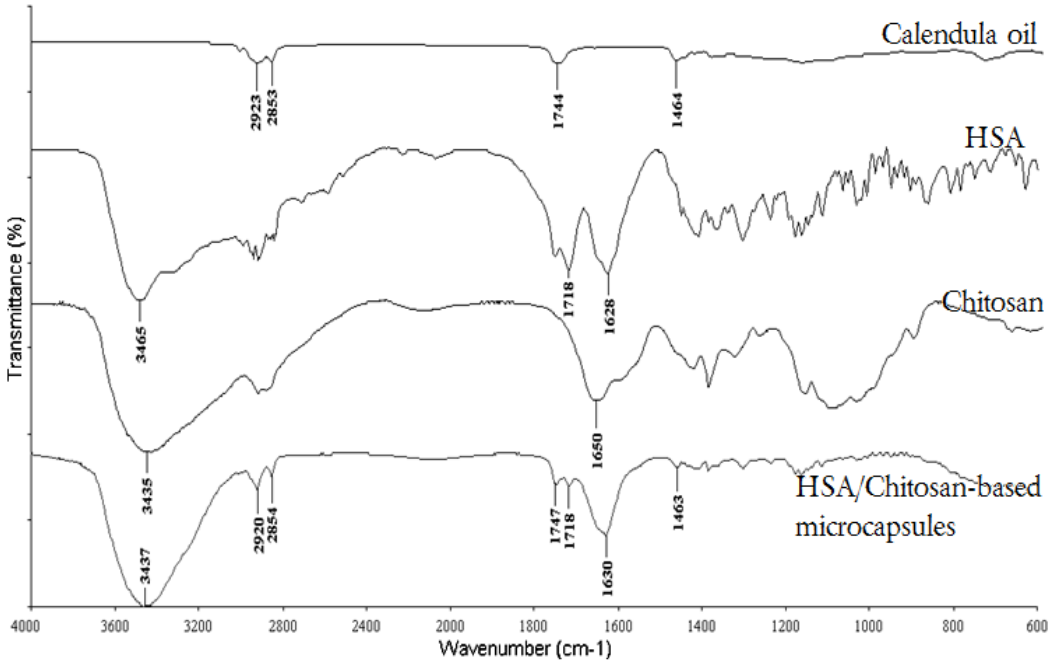


Figure 4.3 FTIR spectrum of HSA/chitosan microcapsules

4.3.4 *In Vitro* Controlled Release of HSA/Chitosan Microcapsules

In the release model of HSA/chitosan microcapsules, the percentage of HSA release was monitored for 12 hours and shown in Figure 4.4. HSA was more readily released from the chitosan-based microcapsules when subjected to the primary release medium of HCl (pH 1.2). It was revealed that approximately half of the drug was released at the first 4 hours in HCl medium simulated the stomach condition in human body. This might be probably due to the fact that the chitosan wall shell of microcapsules was more susceptible to the acidic condition as chitosan was being able to be soluble in acidic medium, resulting in higher leakages of core drugs. HSA was released at a slower speed in the secondary medium of phosphate (pH 7.4) as chitosan wall was more stable and more rigid under alkaline condition. It was observed that approximately 80% of the drug was released after 12 hours.

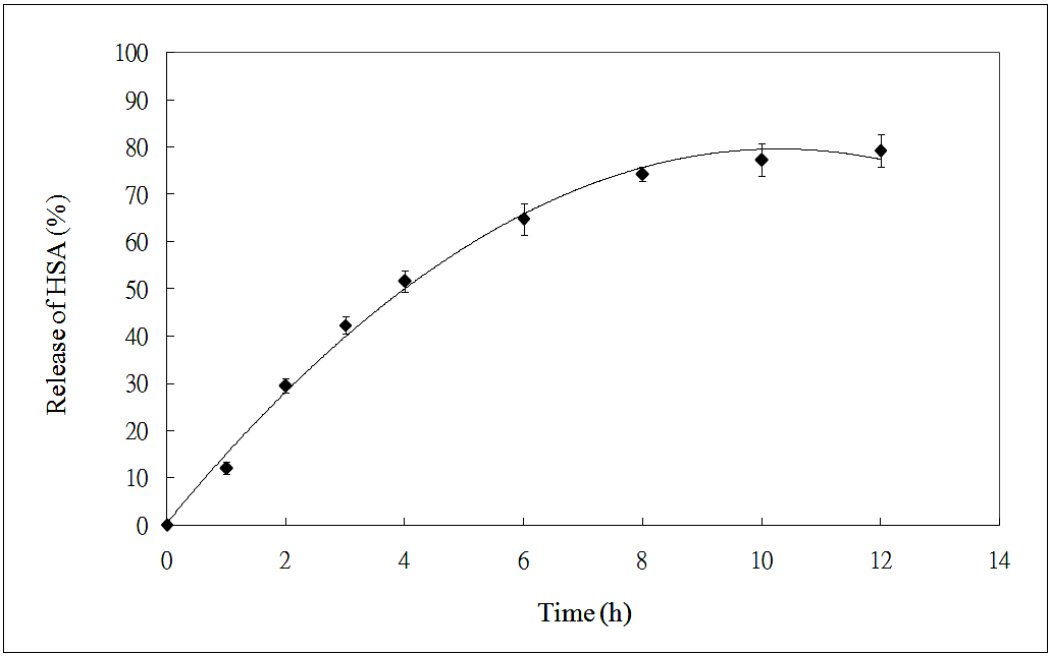


Figure 4.4 Release profile of HSA/chitosan microcapsules in the simulated digestion condition

4.3.5 Effect of HSA/Chitosan Microcapsules on the Plasma ACTH of C57BL/6 Mice Model

The effect of HSA/chitosan microcapsules on the plasma ACTH of C57BL/6 mice model was examined. ACTH level in the blood was measured to help detect, examine and monitor conditions related to excessive or deficient cortisol in the animals. The level of ACTH generally alters in the opposite direction to the level of cortisol. Drugs which induce ACTH to fall include hydrocortisone that act like cortisol. After the treatment period, all the 9 mice survived and no significant change in body weight was observed. From Table 4.1, it was observed that the mean ACTH concentration (pg/mL) in HSA loaded microcapsule mice plasma was about 42.2% lower than that of water control. This might be due to the fact that the HSA loaded microcapsules increased the hydrocortisone level in the blood of the mice. This then reduced the ACTH level in the plasma which is a hormonal negative feedback mechanism.

Table 4.1 ACTH concentration (pg/mL) in mice plasma.

	Water Control	Microcapsule Control	HSA loaded microcapsules
Mice 1	22.42	11.50	17.47
Mice 2	24.75	46.60	23.08
Mice 3	41.45	23.78	10.67
Mean ± SD	29.54 ± 10.38	27.29 ± 17.81	17.07 ± 6.21

4.4 Conclusion

The use of polymeric microcapsules to encapsulate the drug for oral application was believed to be helpful in drug deliveries. Microencapsulated drugs were proposed to be gradually released under control from microcapsules and the sustained release could promote a longer lasting effect of core active drugs. The experiments in this chapter demonstrated that the possibility of oral applications was investigated using the HSA microcapsules. The microencapsulated hydrocortisone has been reported in some studies. Leelarasamee et al. (1986) investigated the controlled release profile of hydrocortisone loaded polylactic acid microcapsules and the effect of different surfactants on the drug release of microcapsules (Leelarasamee *et al.*, 1988). Therefore, this chapter aimed at demonstrating the potentially oral application of microencapsulated HSA using ACTH assay from C57BL/6 mice.

In this study, the mean drug loading efficiency and the mean particle size of HSA/chitosan microcapsules were 53.90% and 3.56μm respectively. The FTIR analysis confirmed the encapsulation of HSA in microcapsules. Approximately 80% of the drug was released from HSA/chitosan microcapsules after 12 hours in the simulated digestion model. The mean ACTH concentration in HSA loaded microcapsule mice plasma was detected to be about 42.2% lower than that of water control. It was speculated that chitosan-based microcapsules could be a versatile drug delivery vehicle for oral medications.

CHAPTER 5

DEVELOPMENT OF 5-FLUOROURACIL/ AND PHYLLANTHIN/ CHITOSAN MICROCAPSULES FOR TOPICAL DRUG DELIVERY

5.1 Introduction

Since the competition in the market is keen, suppliers keep a close eye on developing potential techniques and manufacturing methods for textile finishing so as to search for the new market opportunities. Recently, some patients were found to have allergic response towards intravenous injection while some of them were unable to take medicines by oral. There is a great potential for transdermal drug delivery systems because it provides several possible benefits over the conventional drug delivery routes. These include the minimization of inconvenience and risk during the oral and intravenous administrations, extension of drug effect at a lower dosage level and the easier drug administration resulting in better patient compliance (Elliott, 2003; Krousel-Wood *et al.*, 2005; Ma *et al.*, 2009). Various technologies and methods have been explored for producing the transdermal or topical drug delivery systems. Medicated polymeric microcapsule is one of these ways to deliver the drugs to specific body sites for disease treatments. In accordance with the results generated before, the optimal parameter combination was selected to develop the chitosan-based microcapsules for entrapping two different drugs so as to propose another administration routes for topical drug deliveries.

Skin cancer is one of the most common tumors in Australia and New Zealend. Actinic keratosis can be regarded as a precursor of skin cancer which is the first step in the development of skin cancer. Excessive sun-exposure is the most

important contributing factor for developing actinic keratosis. UV radiation may cause mutations in cellular DNA that may lead to uncontrolled cell growth and proliferation and eventually tumour formation. Actinic keratosis appears as a discrete, dry, rough or scaly lesion. Topical treatment with therapeutic creams is a way for precancerous remedy and skin cancer treatments. 5-Fluorouracil, also known as 5-FU (5-fluoro-1H-pyrimidine-2, 4-dione) is an effective cytotoxic agent used topically to treat various forms of skin cancers. Its active compound revealed an appropriate antitumor activity in the topical treatment of lesions liked squamous cell carcinoma such as actinic keratosis, keratoacanthoma, Browen's disease and superficial epithetliomas of the skin (Ashbel, 1971; Paolina *et al.*, 2008). For the treatment of actinic keratosis, 5-FU can effectively destroy the actinically damaged skin cells and inhibit the excessive cell growth. 5-FU can be topically applied and intravenous injected; but even small dosages may also lead to acute damage of central nervous system.

From the past to the present, medicinal plants were considered useful and helpful to cure the human ailments and diseases. Different parts of medicinal herbs were used to prepare different therapeutic medicines in many countries for many centuries. Phyllanthin is a major bioactive lignan component isolated from the plant extract of *Phyllanthus amarus* which is a family of Euphorbiaceae (Krithika *et al.*, 2009; Chirdchupunseree *et al.*, 2010; Khan *et al.*, 2010). *Phyllanthus amarus* also known as Bhui amla, is a herb which exists in central and south India, the rain forests of Amazon and other tropical areas of the world. This plant has been used in traditional medicine for many years. It has been reported that *Phyllanthus amarus* held the antimicrobial activity towards different microorganisms (Oluwafemi and Debiri, 2008; Chitravadivu *et al.*,

2009) and an antioxidant activity (Krithika *et al.*, 2009; Chirdchupunseree *et al.*, 2010) in some recent studies.

In the present study, it is made use of the microencapsulation technology to encapsulate 5-FU and phyllanthin into chitosan wall shell by a simple coacervation technique so as to enhance the inhibition of 5-FU towards excessive keratinocyte growth and the antioxidant property of phyllanthin towards human fibroblast cells and skin keratinocytes as well as its antimicrobial activity towards *Staphylococcus aureus* respectively. The developed microcapsules containing 5-FU and phyllanthin were suggested to be useful for topical applications.

5.2 Experimental

5.2.1 Materials

Chitosan (75%-85% deacetylated, from Aldrich, Germany) was dissolved in 1% acetic acid. Calendula infused oil was purchased from Easy Creation Asia Limited, Hong Kong. 5-Fluorouracil (5-FU, $C_4H_3FN_2O_2$, molecular weight: 130.08g/mol) and phyllanthin ($C_{24}H_{34}O_6$, molecular weight: 418.53g/mol) were obtained from Sigma-Aldrich, Germany and ChromaDex, USA respectively. Agar and Luria both (LB) were bought from Sigma-Aldrich, Germany. All reagents were purchased from Sigma-Aldrich, Germany.

5.2.2 Preparation of 5-FU/ and Phyllanthin/Chitosan Microcapsules

Twenty milligrams of 5-FU and phyllanthin was firstly dissolved in 2g of calendula infused oil respectively and the synthesis processes were then performed as described in Chapter 3, Section 3.2.2.

5.2.3 Determination of Drug Loading Efficiency

5-FU and phyllanthin loaded microcapsules were dissolved in 0.1N HCl respectively. The samples were then filtered. 5-FU and phyllanthin contents were analyzed by measuring the absorbance at 265nm and 280nm (λ_{max} of drugs in 0.1N HCl) after suitable dilution using UV VIS spectrophotometer (Lambda 18, Perkin Elmer, USA). The drug loading of microcapsules was performed as described previously in Chapter 4, Section 4.2.3. Figure 5.1 shows the the standard calibration curve of 5-FU and Figure 5.2 illustrates the standard calibration curve of phyllanthin.

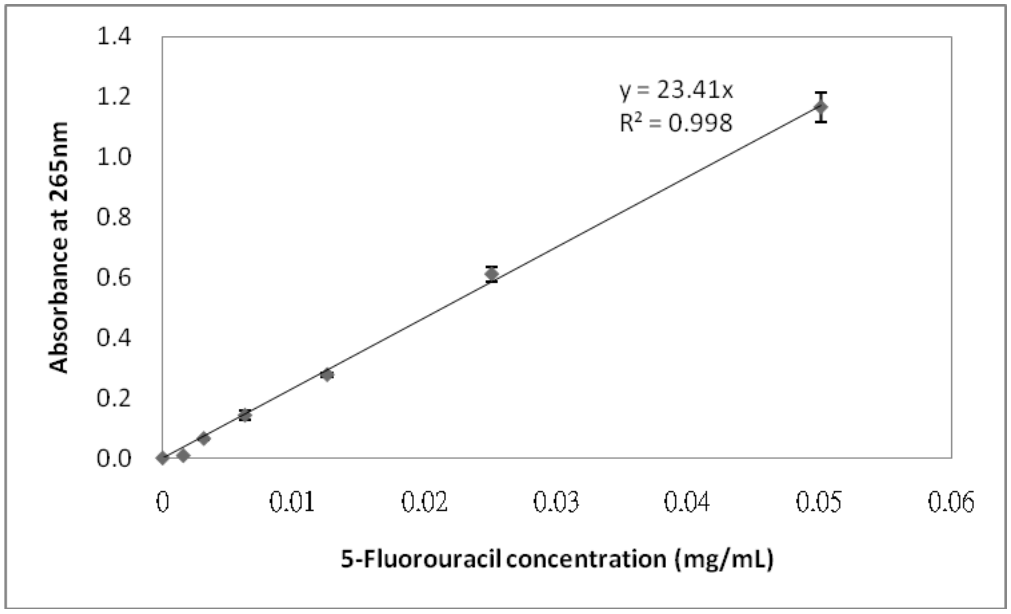


Figure 5.1 Standard calibration curve of 5-FU

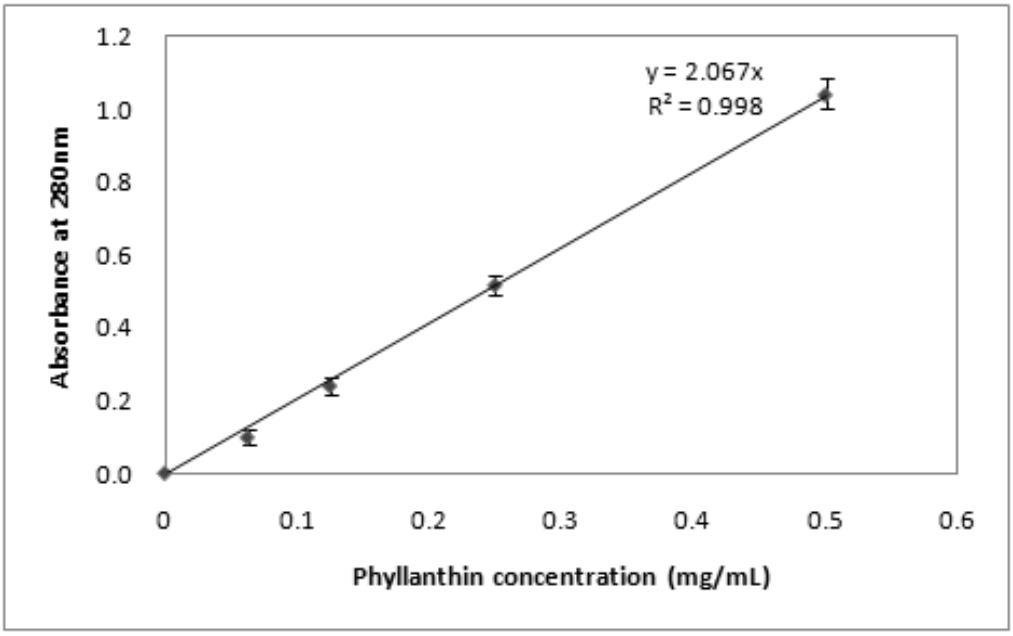


Figure 5.2 Standard calibration curve of phyllanthin

5.2.4 Size of 5-FU/ and Phyllanthin/Chitosan Microcapsules

The particle size of drug-loaded microcapsules was investigated as described in Chapter 3, Section 3.2.4.

5.2.5 Morphological Analysis of 5-FU/ and Phyllanthin/Chitosan Microcapsules

Surface Morphology of drug-loaded microcapsules was evaluated as described in Chapter 3, Section 3.2.5.

5.2.6 FTIR Analysis of 5-FU/ and Phyllanthin/Chitosan Microcapsules

FTIR spectrum of drug-loaded microcapsules was analyzed as described in Chapter 3, Section 3.2.6.

5.2.7 *In Vitro* Controlled Release of 5-FU/ and Phyllanthin/Chitosan Microcapsules

A given amount of 5-FU/ and phyllanthin/chitosan microcapsules was suspended in a phosphate solution pH 7.4 medium for 72 hours and 120 hours at room temperature and stirred at 100rpm respectively. Aliquots were taken at desired time intervals. The drawn sample was filtered using filter paper and the residue was then returned to the suspension medium. The clear filtrate of 5-FU/ and phyllanthin/chitosan microcapsules was analyzed at λ_{max}= 265nm and 280nm respectively by UV VIS spectrophotometer (Lambda 18, Perkin Elmer, USA) for the determination of drug content. Each sample was conducted in triplicate.

5.2.8 Inhibitory Effect of 5-FU/Chitosan Microcapsules on the HaCaT Keratinocytes

In order to investigate the keratinocyte inhibitory effect of 5-FU containing chitosan microcapsules, the microcapsules were conducted on human skin keratinocytes. Sulforhodamine B assay was used for cell viability evaluation. Human cells including HaCaT skin keratinocytes were removed from sterile cell culture flasks with trypsin and neutralized with fetal bovine serum. After washing with phosphate buffered saline and centrifugation, skin cells were re-suspended in complete cell culture medium at a concentration of approximately 1×10^5cells/mL and counted manually using a haematocytometer under an inverted microscope. Human cells seeded in the microtitre plates for 24 hours were prepared for the screening of our microcapsules. Microcapsules at 100μg/mL of 5-FU, blank microcapsules without drugs and 100μg/mL of 5-FU were added respectively and incubated with cells for a further period of 96 hours.

Afterwards, the evaluation of possible inhibitory potential was performed by the sulforhodamine B protein staining methods. Firstly, skin cells were fixed with trichloroacetic acid, washed with distilled water and stained with sulforhodamine B. Afterwards, cells were washed again with acetic acid and stained cells were dissolved in unbuffered Tris-base. Finally, optical absorptions were measured at 575nm using a microplate reader (Victor V form Perkin Elmer, Life Sciences).

5.2.9 *In Vitro* 5-FU Delivery Model Using Nude Mice Skin

Nude mice were sacrificed and autopsy was performed to obtain the skin immediately. They were stored under ice condition in order to keep them fresh. The skin was immersed into the cell culture medium. The microcapsules containing 100μg/cm^2 of 5-FU and microcapsules without drugs were fabricated on the cotton fabrics respectively. The microcapsule treated cotton samples were then fixed on the skin at 25˚C for 24 hours. The cotton fabrics were removed carefully and they were used for further release analysis. The suspended oil attached on the surface of the skin was washed with PBS buffer and used for further visual assessment.

5.2.10 Determination of Reactive Oxygen Species of Phyllanthin/Chitosan Microcapsules

Human fibroblast cells and human keratinocytes were seeded at a concentration of approximately 1 × 10^5cells/mL and counted manually using a haematocytometer under an inverted microscope. After 24 hours, the culture medium was changed and incubated with microcapsules containing 50μg of phyllanthin, blank microcapsules and untreated control. After a further incubation of 24 hours, the culture medium was changed and cells were

incubated with 2′,7′-dichlorofluorescein diacetate (Molecular Probe) for a further of 30 minutes. Both cells were washed and collected. Cells were subsequently lysed and total cellular protein was collected while debris was discarded. Protein content was determined by using the Bradford reagent. The relative level of Reactive Oxygen Species (ROS) from each of 20μg of total protein sample was determined by measuring its fluorescence units at 515nm after an excitation at 485 nm using a microplate reader.

5.2.11 Antibacterial Assessment of Phyllanthin/Chitosan Microcapsules

Staphylococcus aureus (S. aureus) (kindly provided by Dr. R.S.M. Wong, Haematology Division and Toxicology Division, Department of Medicine and Therapeutics, Prince of Wales Hospital) was used to study the effectiveness of phyllanthin loaded chitosan microcapsules against its growth in culture. *S. aureus* were diluted with broth medium and plated on preset agar plate. One hundred percent of cotton fabrics were cut in circular shape with 1cm in diameter. Microcapsules consisting 150μg and 300μg phyllanthin, 150μg and 300μg phyllanthin, blank microcapsules without drugs (as negative control) and methicillin (as positive control) on the fabric strip were placed on the surface of the agar. The plates were placed in incubator at 37˚C for 24 hours and growth inhibition of *S. aureus* on the agar plates were recorded according to our scheduled time interval.

5.3 Results and Discussion

5.3.1 Drug Loading and Particle Size

The mean drug loading efficiency and drug loading of 5-FU/chitosan microcapsules was examined to be 50.15% ± 1.45 and 1223.10μg/mL ± 35.30 respectively while those of phyllanthin microcapsules were 60.07% ± 3.85 and 706.66μg/mL ± 45.27 respectively. The particle size of 5-FU/chitosan microcapsules ranged from 1.59 to 5.45μm with the mean of 3.21μm while those of phyllanthin microcapsules ranged from 2.16 to 7.29μm and the mean particle size was 5.32μm.

5.3.2 Surface Morphology

The SEM images of 5-FU/ and phyllanthin/chitosan microcapsules were shown in Figure 5.3 and 5.4 respectively. It was observed that the developed microcapsules were approximately spherical in shape with relatively smooth surface morphology.

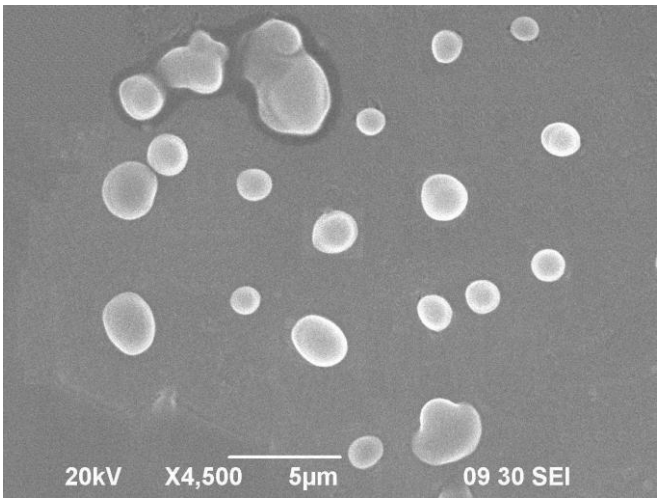


Figure 5.3 SEM image of 5-FU/chitosan microcapsules

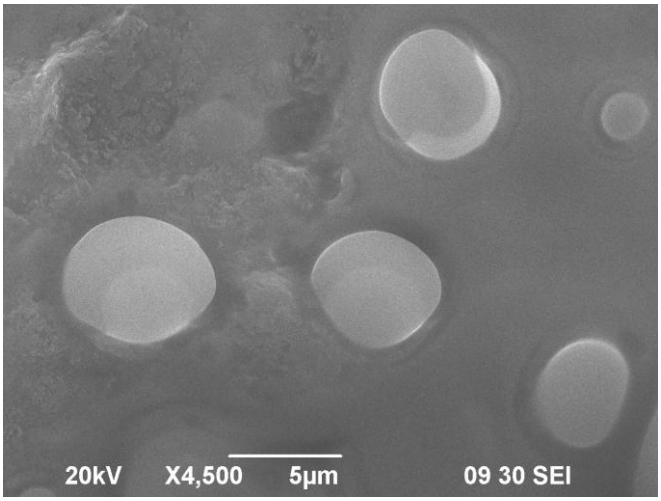


Figure 5.4 SEM image of phyllanthin/chitosan microcapsules

5.3.3 FTIR Spectrum

The FTIR spectra of 5-FU/ and phyllanthin/chitosan microcapsules are shown in Figure 5.5 and 5.6 respectively. Significant peaks were observed in Chitosan spectrum at 3435cm^{-1} and 1650cm^{-1} which were related to hydrogen-bonded O-H stretching and C=O stretching (amide I) respectively. Calendula infused oil had the significant peaks at 2853cm^{-1} and 2923cm^{-1} (C-H aliphatic stretching), 1744cm^{-1} (C=O stretching) and 1464cm^{-1} (C=C stretching). Significant peaks were observed in 5-FU spectrum at 3433cm^{-1} (N-H stretching), 1639cm^{-1} (C=O stretching), 1251cm^{-1} (C=C stretching) and 1180cm^{-1} (C-F bond) (Yan *et al.*, 2011). Phyllanthin exhibited the significant peaks at 1026cm^{-1} (C-O group) and 1635cm^{-1} (C=C group).

The 5-FU loaded chitosan microcapsule spectrum (Figure 5.5) demonstrated the specific peaks of 5-FU, chitosan and calendula infused oil. The absorbance at λ = 3436cm^{-1} in microcapsule spectrum showed the existence of the O-H stretching from chitosan and the N-H stretching from 5-FU that superimposed with each

other. The C=O stretching at 1642cm^{-1} which occurred in microcapsule spectrum also revealed the presence of chitosan and 5-FU that laid over each other. The absorbance at λ = 1254cm^{-1} (C=C stretching) and λ =1172cm^{-1} (C-F bond) detected the occurrence of 5-FU in microcapsule spectrum. It was also perceived that the peaks of calendula infused oil from the C-H aliphatic stretching at 2853cm^{-1} and 2929cm^{-1} were existed in the spectrum of the developed microcapsules. The C=O stretching at 1742cm^{-1} and the C=C stretching at 1464cm^{-1} showed the existence of calendula infused oil in the developed microcapsules. The overall results confirm the synthesis of 5-FU loaded chitosan microcapsules.

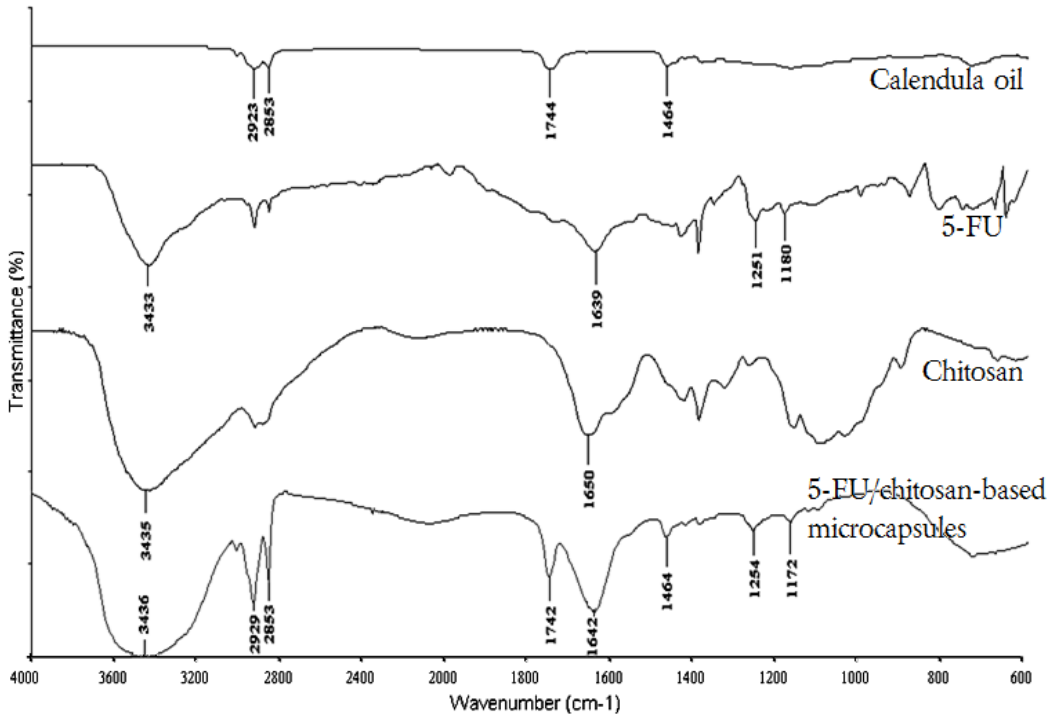


Figure 5.5 FTIR spectrum of 5-FU/chitosan microcapsules

The spectrum of phyllanthin loaded chitosan microcapsules (Figure 5.6) showed the characteristic peaks of phyllanthin, chitosan and calendula infused oil. The

absorbance at 3435cm^{-1} in microcapsule spectrum showed the existence of the O-H from chitosan wall while the peak at 1634cm^{-1} revealed the presence of C=O stretching (amide I) from chitosan and C=C group from phyllanthin. The absorbance at λ = 1026cm^{-1} occurred in microcapsule spectrum referring the C-O group from phyllanthin. It was observed that the absorbance of calendula infused oil from the band of C-H aliphatic stretching at λ = 2853cm^{-1} and λ = 2923cm^{-1} existed in the spectrum of the developed microcapsules. The band of the C=O stretching (carboxylic acid ester) at 1746cm^{-1} and the C=C stretching at 1464cm^{-1} revealed the presence of calendula infused oil in the drug loaded microcapsules. The FTIR results showed that the developed microcapsules contained the chemical ingredients and functional groups of the wall material (chitosan) and the core active ingredient (phyllanthin dissolved in calendula infused oil). The overall results confirm the development of phyllanthin loaded chitosan microcapsules.

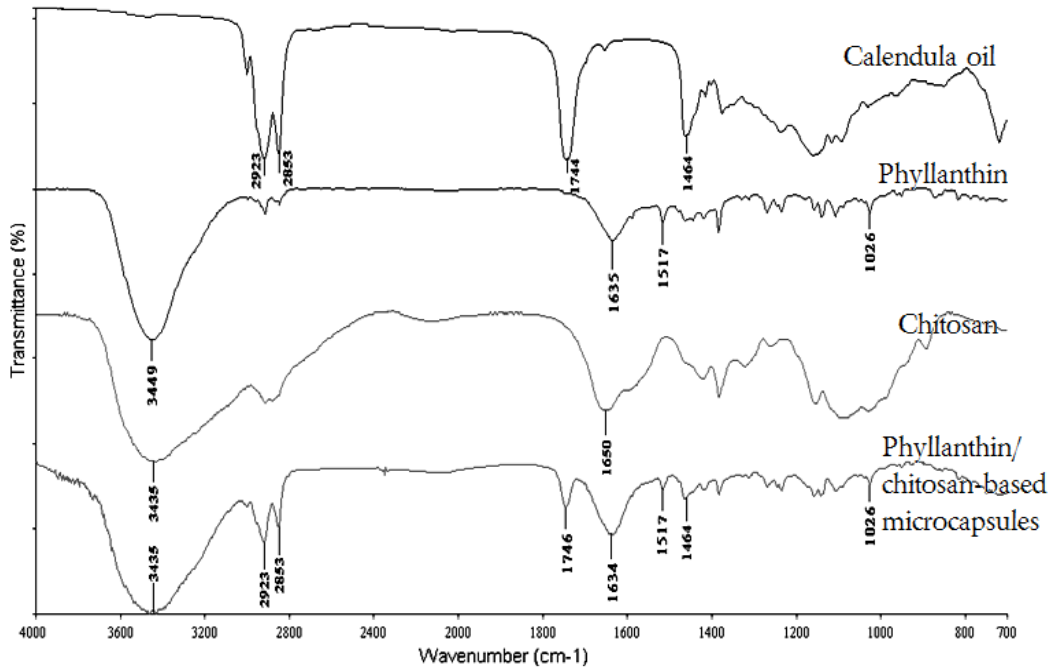


Figure 5.6 FTIR spectrum of phyllanthin/chitosan microcapsules

5.3.4 *In Vitro* Controlled Release of 5-FU/ and Phyllanthin/Chitosan Microcapsules

In the release model of 5-FU/chitosan microcapsules, the percentage of drug release was investigated for 72 hours and shown in Figure 5.7. 5-FU was released slowly in the phosphate (pH 7.4) medium. In the first 6 hours, more than 20% of 5-FU was released from the chitosan microcapsules. After 24 hours, another 20% of the drug was released where 48 hours and 72 hours were closed to the plateau level.

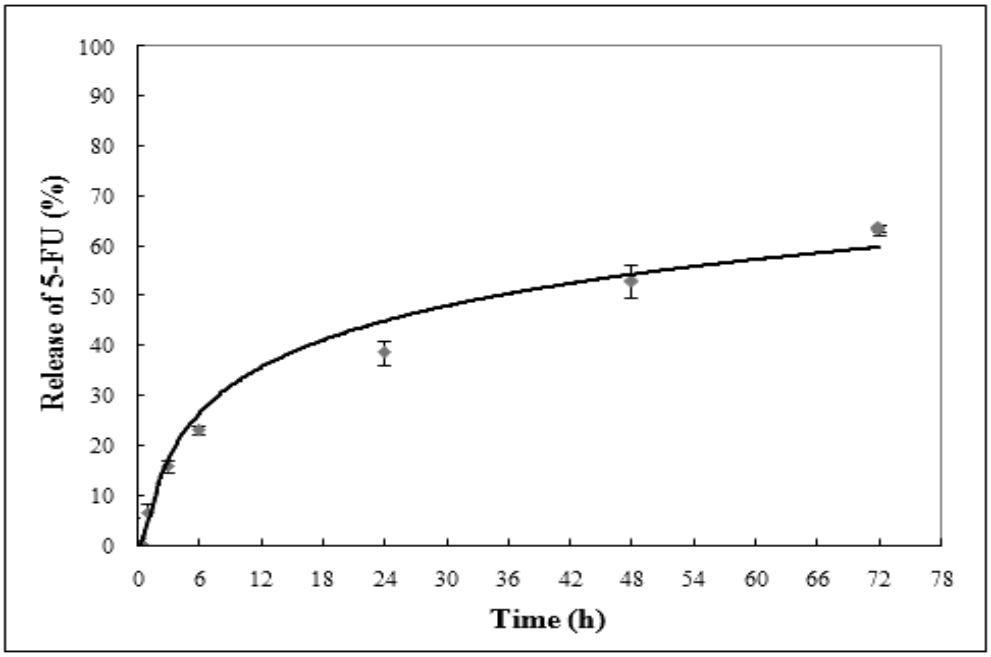


Figure 5.7 Release profile of 5-FU/chitosan microcapsules in the simulated skin condition

In the *in vitro* release model of phyllanthin loaded chitosan microcapsules, the percentage of drug release was investigated for 120 hours and shown in Figure 5.8. Phyllanthin was gradually released in the phosphate (pH 7.4) medium. After the first 24 hours, it was observed that approximately 20% of the drug was

released from chitosan microcapsules. After 72 hours, another 25% of the drug was slowly released. The drugs were further released from the microcapsules and there was more than 60% of phyllanthin released after 120 hours.

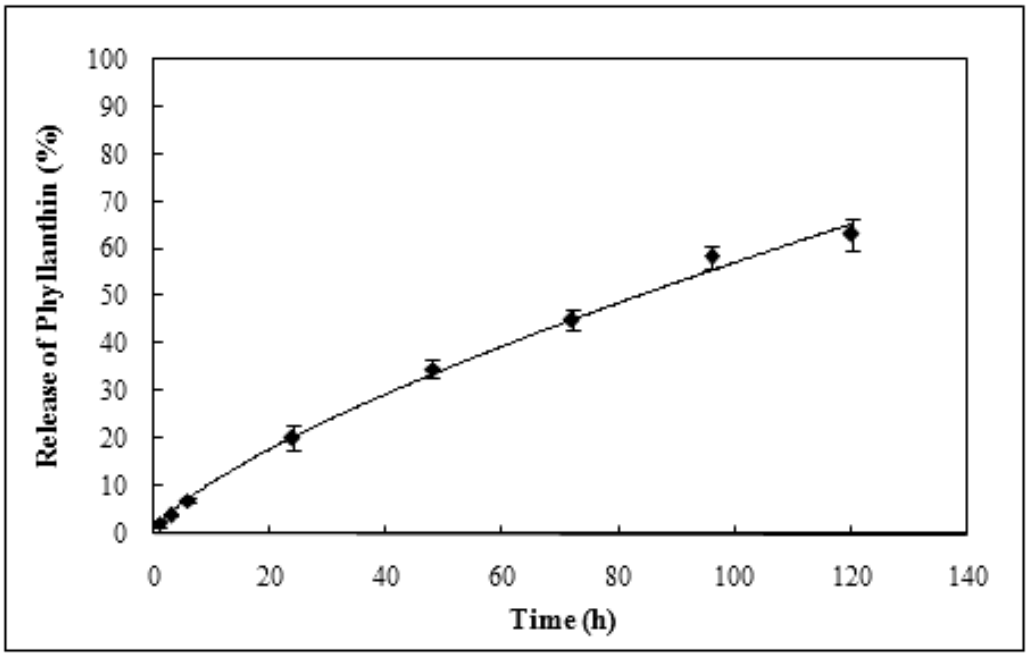


Figure 5.8 *In vitro* release of phyllanthin/chitosan microcapsules in the simulated skin condition

5.3.5 Inhibitory Effect of 5-FU/Chitosan Microcapsules on the HaCaT Keratinocytes

Figure 5.9 shows the inhibition of 5-FU containing microcapsules towards human skin keratinocytes. It was demonstrated that both untreated control (Figure 5.9A) and blank microcapsules without drugs (Figure 5.9B) exhibited a high integrity of cellular structure while both 5-FU microcapsules (Figure 5.9C) and 5-FU (Figure 5.9D) possessed certain damages of cellular structure. From Figure 5.9E, it was observed that blank chitosan microcapsules without drugs had a relatively higher proliferation rate of keratinocytes when compared with

untreated control. The growth inhibitory potency of 5-FU microcapsules and 5-FU showed significant differences as compared to untreated control. Microcapsules containing 100μg/mL of 5-FU revealed a stronger growth inhibition of skin keratinocytes when compared with that of 5-FU. The sustained release of 5-FU from microcapsules might promote a continuous growth inhibition towards skin keratinocytes. It was speculated that the chitosan microcapsules might be an effective carrier for drug delivery.

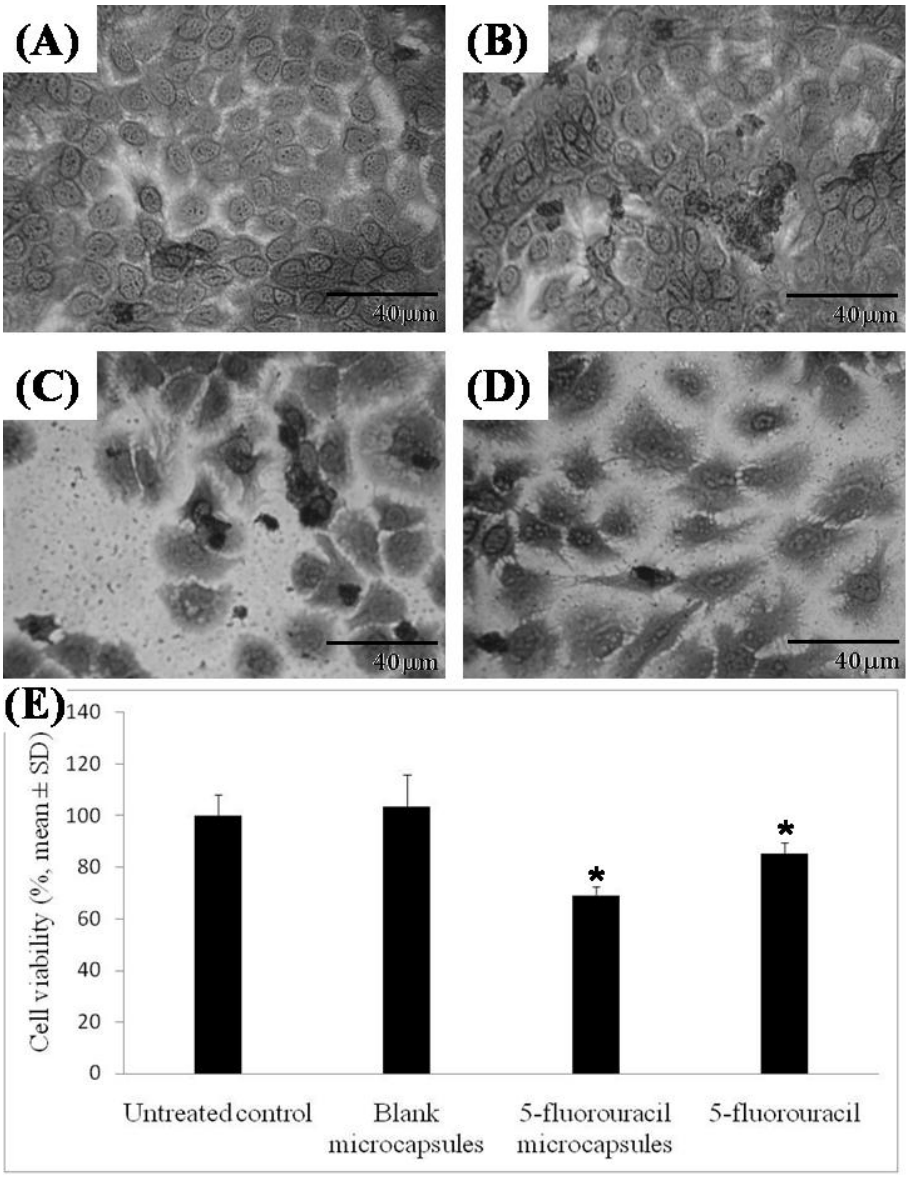


Figure 5.9 Growth inhibition towards skin keratinocytes: (A) untreated control, (B) blank microcapsules, (C) 5-FU microcapsules, (D) 5-FU, and (E) percentage of cell viability. Each experiment was done in triplicate and a mean value was obtained while three independent experiments were performed. Results are shown as mean ± SD from their mean values of these three independent experiments. T-test shows that there were significant differences between untreated control and 5-fluorouracil microcapsules; untreated control and 5-fluorouracil; as well as 5-fluorouracil microcapsules and 5-fluorouracil (*p<0.05)

5.3.6 *In Vitro* 5-FU Delivery Model Using Nude Mice Skin

From Figure 5.10, it was observed that the 5-FU of microcapsule fabricated cotton sample was delivered to the nude mice skin though the oil carrier. The released content of 5-FU was further demonstrated. The mean release of 5-FU from microcapsule treated cotton fabrics was 33.38% ± 9.93 and this result was consistent with the *in vitro* release experiment.

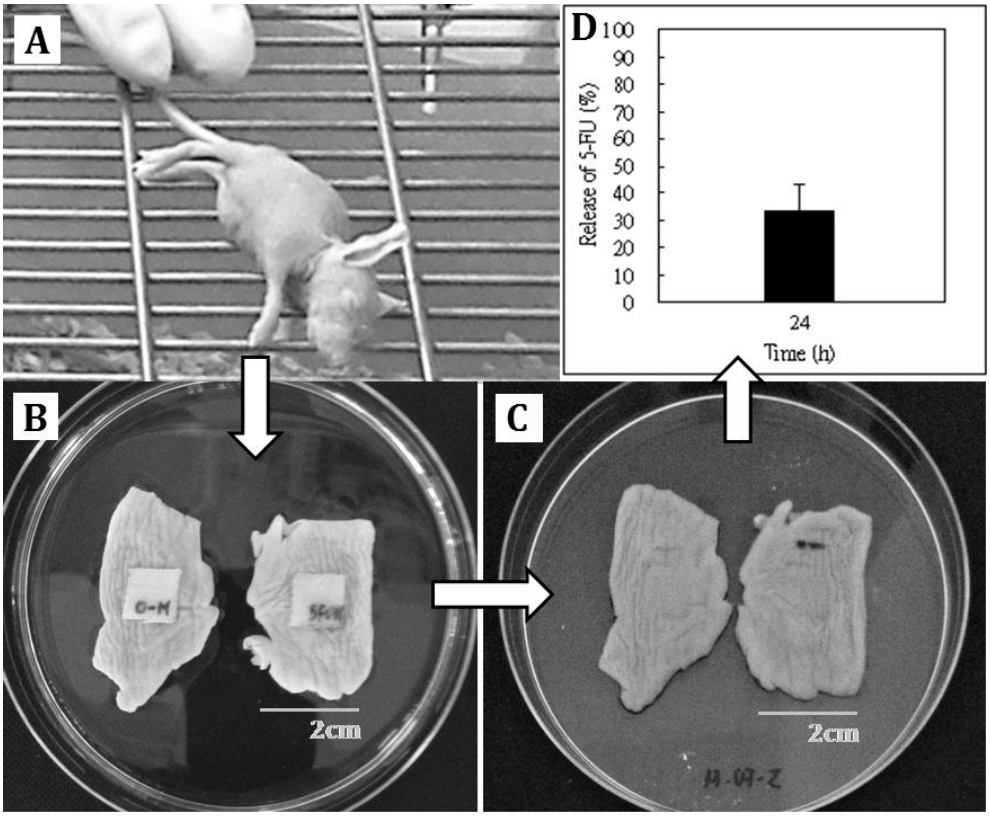


Figure 5.10 Nude mice skin experiment was performed to demonstrate the possible delivery of 5-FU from microcapsule fabricated cotton: (A) nude mouse, (B) nude mouse skin treated with microcapsule fabricated cotton samples (right hand side: 5-FU microcapsules treated cotton; left hand side: blank microcapsules treated cotton), (C) nude mouse skin treated with microcapsule fabricated cotton after 24h, and (D) release of 5-FU from microcapsule fabricated

cotton after 24h. For (B), and (C), the left sample represents the microcapsule without drugs and the right sample represents the 5-FU loaded microcapsules. The data in panel (D) represent the mean from *in vitro* release of 5-FU loaded microcapsule experiments ± SD from three independent experiments

5.3.7 Determination of Reactive Oxygen Species of Phyllanthin/Chitosan Microcapsules

The relative percentage of generation of reactive oxygen species (ROS) as detected by 2'7'-dichlorofluorescein diacetate from human fibroblast cells and human keratinocytes was shown in Table 5.1. The results revealed that phyllanthin possessed a better antioxidant property as compared to the untreated control. ROS produced from human cells are used for signaling and maintaining the cell growth. However, excessive ROS could be harmful to the cells and this could also induce the cell aging. Phyllanthin has been reported to be an effective antioxidant agent which contained the free radical scavenging activity of phyllanthin by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay and protected the HepG2 hepatocellular cancer cells from CCl_4-induced toxicity (Krithika *et al.*, 2009). It was also observed that phyllanthin containing microcapsules exhibited a better inhibition of ROS from human fibroblast cells and keratinocytes when compared with that of phyllanthin. This might be due to the sustained release of phyllanthin from microcapsules which could continuously reduce the amount of endogenous ROS from human fibroblasts and keratinocytes.

Table 5.1 Relative percentage of generation of ROS as detected by 2'7'-dichlorofluorescein diacetate from human fibroblast cells and human keratinocytes

	Control	Phyllanthin	Phyllanthin loaded microcapsules
Fibroblast	100 ± 4.13	86.05 ± 4.57	68.04 ± 1.25
Keratinocyte	100 ± 9.03	78.13 ± 4.66	70.18 ± 2.13

Results are shown as mean ± SD from three independent experiments.

5.3.8 Antibacterial Assessment of Phyllanthin/Chitosan Microcapsules

Figure 5.11 shows that the microcapsules containing 300μg phyllanthin slightly improved the anti-*staphylococcus aureus* (*S. aureus*) activity when compared to that of phyllanthin. Microcapsules containing 150μg phyllanthin showed comparable growth inhibition towards *S. aureus* as the same dose of the free drug (Figure 5.11A). The zone of clearance of both phyllanthin containing microcapsules and phyllanthin was shown in Table 5.2. It was observed that higher concentration of phyllanthin in microcapsules exhibited stronger antibacterial activity towards *S. aureus*. However, there was insignificant difference in growth inhibition of phyllanthin in the form of free drug towards *S. aureus*. All phyllanthin containing microcapsules and phyllanthin exhibited the clear contact areas.

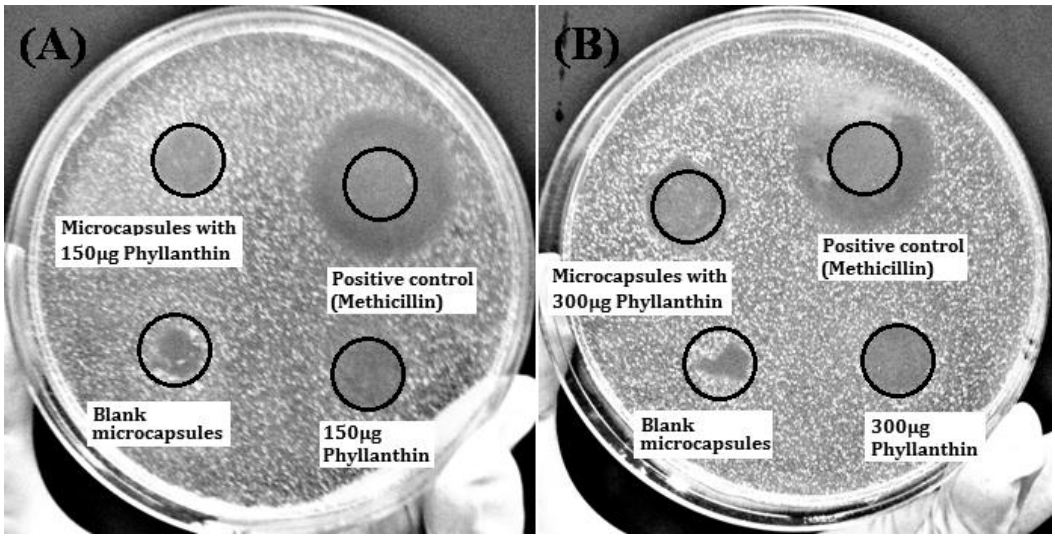


Figure 5.11 Growth inhibition towards *S. aureus*. Methicillin was used as the positive control and blank microcapsules without drugs were used as the negative control

Table 5.2 Growth inhibitory study of phyllanthin containing microcapsules and phyllanthin towards *S. aureus*

	Blank microcapsules	Microcapsules containing 150µg Phyllanthin	Microcapsules containing 300µg Phyllanthin	150µg Phyllanthin	300µg Phyllanthin	Positive control (Methicillin)
Contact area	Semi-clear	Clear	Clear	Clear	Clear	Clear
Zone of clearance (Mean, mm)	Nil	Nil	2	Nil	Nil	8

Each test was performed in triplicates.

5.4 Conclusion

Dubey and Parikh (2004) developed chitosan microspheres containing 5-FU and evaluated their *in vitro* release performance. However, few studies have focused on studying the possibility of topical 5-FU loaded microcapsules (Dubey and Parikh, 2004). Therefore, we aimed to demonstrate the potentially topical applications of microencapsulated 5-FU using *in vitro* human skin keratinocytes

as well as nude mice skin delivery model respectively. With respect to the antimicrobial activity, the study is in line with several reports, including the recent study of Guarda *et al.* (2011) which aimed at determining the antimicrobial activity properties of plastic flexible films with a coating of microcapsules containing carvacrol and thymol as natural antimicrobial activity agents. Microencapsulation of these agents enabled their controlled release and led to growth inhibition of a broad spectrum of microorganisms such as, *Escherichia coli, Staphylococcus aureus, Listeria innocua, Saccharomyces cerevisiae* and *Aspergillus niger*. Interestingly, these authors found a synergistic effect of combinations of thymol and carvacrol. The antimicrobial effect of capsaicin microcapsules for potential food storage applications (Xing *et al.*, 2006) was also reported. The microcapsules containing capsaicin exhibited anti-*Botrytis cinerea* and anti-*Aspergillus niger* activities. These studies encourage further research aimed at identifying possible combinations of phyllanthin with other antimicrobial activity agents delivered by microcapsules.

To conclude, the chitosan-based microcapsules containing 5-FU and phyllanthin have been developed by a simple coacervation method. The 5-FU and phyllanthin containing chitosan microcapsules exhibited the mean drug loading efficiency of approximately 50% and 60% respectively and the mean particle size of 5-FU/ and phyllanthin/chitosan microcapsules was about 3μm and 5μm respectively. Both drug containing microcapsules were spherical in shape with smooth surface morphology. The entrapment of 5-FU and phyllanthin into the chitosan microcapsules was confirmed by the FTIR analysis. The controlled release study demonstrated that the drugs were released from both microcapsules at a gradual rate. It was also revealed that microcapsules containing 100μg/mL of

5-FU leaded to a better cell growth inhibition of human keratinocytes when compared to that of 5-FU. *In vitro* drug delivery model also demonstrated that 5-FU could be released from microcapsule treated textiles on the intact skin model. *In vitro* biological assays revealed that these phyllanthin containing microcapsules showed a stronger anti-oxidation potential on both human fibroblasts and keratinocytes as well as a better growth inhibitory activity towards *S. aureus*. Therefore, it was speculated that chitosan-based microcapsules could be a versatile drug delivery vehicle for topical medications.

CHAPTER 6

OPTIMIZATION OF FORMALDEHYDE-FREE AGAR/GELATIN - BASED MICROENCAPSULATION SYSTEM FOR BERBERINE AND GALLIC ACID DELIVERIES

6.1 Introduction

Nowadays, the safety issue on biological applications of biomedical remedies is the major concern. Drug delivery associated with microencapsulation technology is under consideration in many laboratories. It is because microencapsulated drugs are believed to offer a better therapeutic performance on human absorption and promote the controlled release of drugs. The potential topical and oral applications of microcapsules containing biomedical agents or drugs have been reported. The microcapsules were commonly prepared by the crosslinking technique using gelatin as the wall matrix or shell material. Gelatin, as a biodegradable polymer, is essential to be crosslinked to modulate the general characteristics of microcapsules, including enhancement of the wall shell or wall matrix of microcapsules. The most popular crosslinker for gelatin microcapsulation is formaldehyde despite the fact that it is considered toxic to the human body.

After the inhalation, formaldehyde is rapidly converted to hydroxymethylglutation by formaldehyde-glutathione conjugate. Hydroxymethylglutation is further changed to formate by formaldehyde dehydrogenase which is widely distributed in mammalian tissues such as liver and red blood cells in humans. This is the major metabolic enzyme included in the metabolism of formaldehyde. The carbon atom in formate is then oxidized to carbon dioxide or incorporated into nucleotide bases including purines,

thymidine as well as amino acids via tetrahydrofolate-dependent one-carbon biosynthetic pathways. As a result, formaldehyde is eliminated from the body as formate in urine or CO_2 in expired air. However, if formaldehyde is not metabolized by formaldehyde dehydrogenase, it can form crosslinkages between proteins and single-stranded DNA resulting in endogenous and exogenous formaldehyde entry in the formaldehyde dehydrogenase metabolic pathway (Heck *et al.*, 1985; Norliana *et al.*, 2009). The United States Environmental Protection Agency (EPA) has established for formaldehyde a maximum daily dose reference (RfD) of 0.2 mg kg^{-1} body weight per day. Following exposures increasingly greater than the RfD, the potential for adverse health effects increases (Wang *et al.*, 2007). Human studies showed that chronic inhalation exposure to formaldehyde can cause allergy in eyes and mucous membranes of the respiratory system, in relation to nose and throat. Clinical studies demonstrated that eyes, nose and throat irritation was experienced in volunteers after exposure to formaldehyde ranging from 0.25-3.0 ppm (Kulle, 1993; Pazdrak *et al.*, 1993) Textiles containing formaldehyde can also lead to allergic contact dermatitis (O'Quinn and Barrett Kennedy, 1965).

To solve the problems of applying formaldehyde, a simple and useful method is to employ agar associated with gelatin as the wall matrix materials in the formation of microcapsules. Agar is a natural polysaccharide extracted from seaweed, mainly composed of agarose and agaropectin. Agar molecules appear in random coils when they are dissolved in hot water at high temperature (>85˚C), while they are supposed to form double helices, which subsequently crosslink together with gelatin molecules to give a gel network at low temperature (<30˚C).

Berberine is a quaternary alkaloid isolated from natural plants originated from Berberidaceae, Coptis, Fumariaceae, Mahonia, Papaveraceae and other herb species that have traditionally been used in Chinese herbal medicine for many years. Berberine has also been proven to have antibacterial properties (Cernakova and Kostalova, 2002; Jin *et al.*, 2010; Zhang *et al.*, 2011). Gallic acid (3, 4, 5-trihydroxybenzoic acid) is an effective natural antioxidant agent originated from the plant kingdom, commonly present in phenolic materials such as tannins, flavonoids and lignin that offer the plants with protection against the oxidative reaction and free radicals (Slawinska *et al.*, 2007). It was proposed that gallic acid exhibits protective effects over the oxidative damage in rat liver and kidney (Rasool *et al.*, 2010; Vijaya Padma *et al.*, 2011).

In this study, agar together with gelatin as the wall matrix materials were employed to prepare the microencapsulation system by the crosslinking method, and focused to investigate the optimal condition parameters of producing agar/gelatin-based microcapsules containing berberine and gallic acid in terms of the ratio of agar to gelatin, the ratio of polymer to oil, the ratio of oil to surfactant and the stirring speed. The determination of an optimal condition for producing drugs containing agar/gelatin microcapsules as the drug delivery model was mainly based on the drug loading efficiency (first priority) and the mean particle size (second priority). Afterwards, berberine and gallic acid loaded agar/gelatin microcapsules were employed to demonstrate the feasibility of the developed microencapsulation system for both topical and oral drug deliveries.

6.2 Experimental

6.2.1 Materials

Agar, gelatin, berberine were obtained from Sigma-Aldrich, Germany. Gallic acid was obtained from the China National Institute for the Control of Pharmaceutical and Biological Products. One hundred percent of pure olive oil was purchased from Easy Creation Asia Limited, Hong Kong. Pepsin from porcine gastric mucosa (Sigma-Aldrich, Germany), Trypsin from porcine pancrease (Sigma-Aldrich, Germany) and Amino Lipase A from *Aspergillus niger* (Aldrich, USA) were applied to the *in vitro* release test. Agar and Luria both (LB) were bought from Sigma-Aldrich, Germany. All the chemical reagents were supplied from Sigma-Aldrich, Germany.

6.2.2 Preparation of Berberine and Gallic Acid Loaded Agar/Gelatin Microcapsules

Agar, gelatin and drugs in aqueous form were firstly hydrated in deionized water. The agar solution and the gelatin solution were mixed together using a magnetic stirrer (Heidolph – MR 3001, Germany) at 1000 rpm for 10 minutes of 50˚C to 70˚C. The drug solution was then poured into the agar-gelatin mixture and mixed continuously for 5 minutes. The 100% pure olive oil with Span 80 as a surfactant was added to the agar-gelatin-drug mixture followed by continuous stirring to from the water-in-oil emulsion. The emulsion was subsequently subjected to a homogenizer (BioSpec Products, USA) in order to form the homogeneous emulsion. The emulsion was allowed to cool down until it was below 25˚C and continuously stirred for 3 hours. The precipitates were then washed by acetone, followed by deionized water. The resulting microcapsules were suction-filtered and stored in the desiccator. Figure 6.1 shows the flow diagram of microcapsule

formation process.

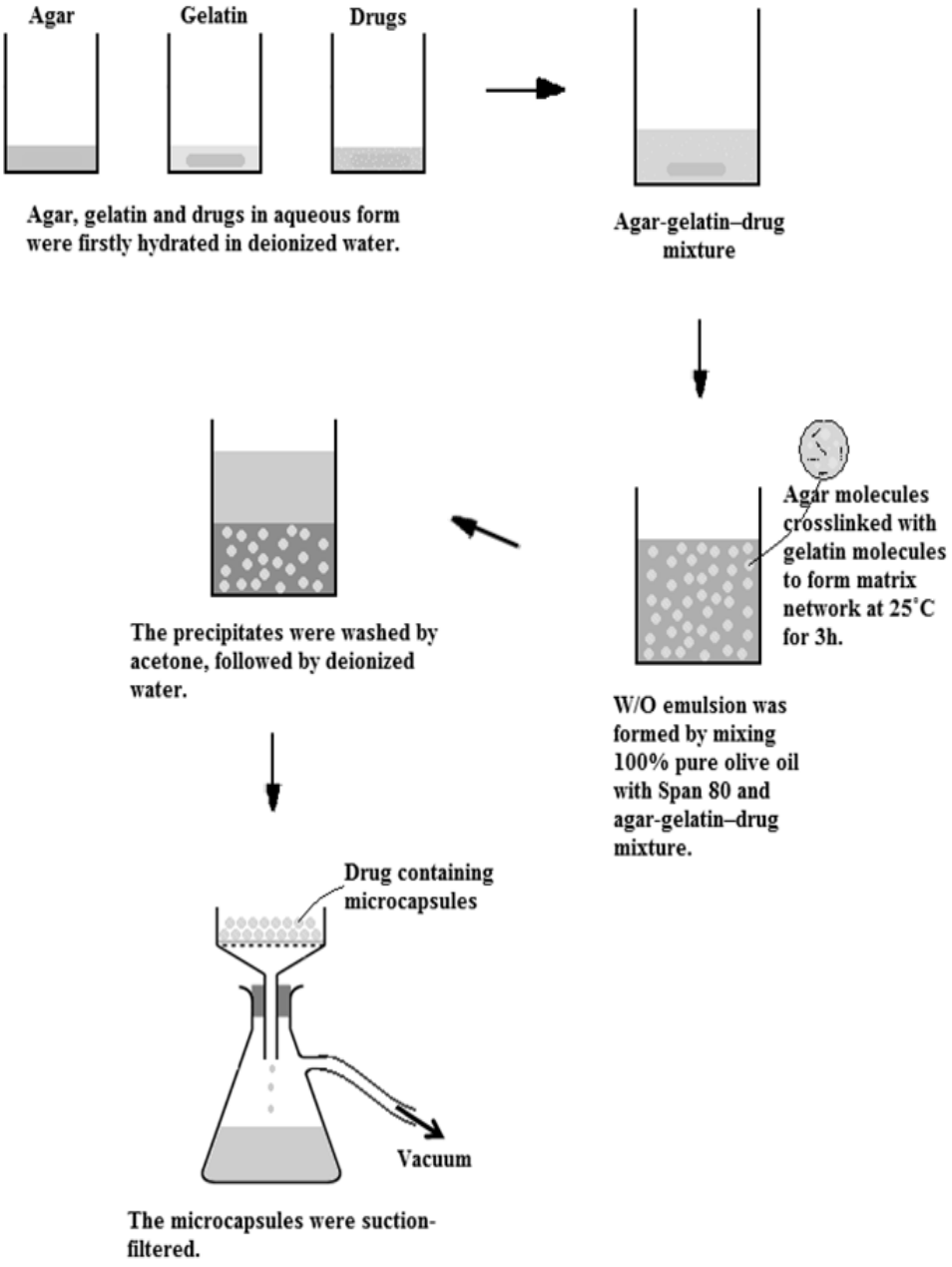


Figure 6.1 Flow diagram of agar/gelatin-based microcapsules preparation

Figure 6.2 shows the images of agar powder, gelatin powder, berberine and berberine loaded agar/gelatin microcapsules and Figure 6.3 illustrates the images of agar powder, gelatin powder, gallic acid and gallic acid loaded agar/gelatin

microcapsules.

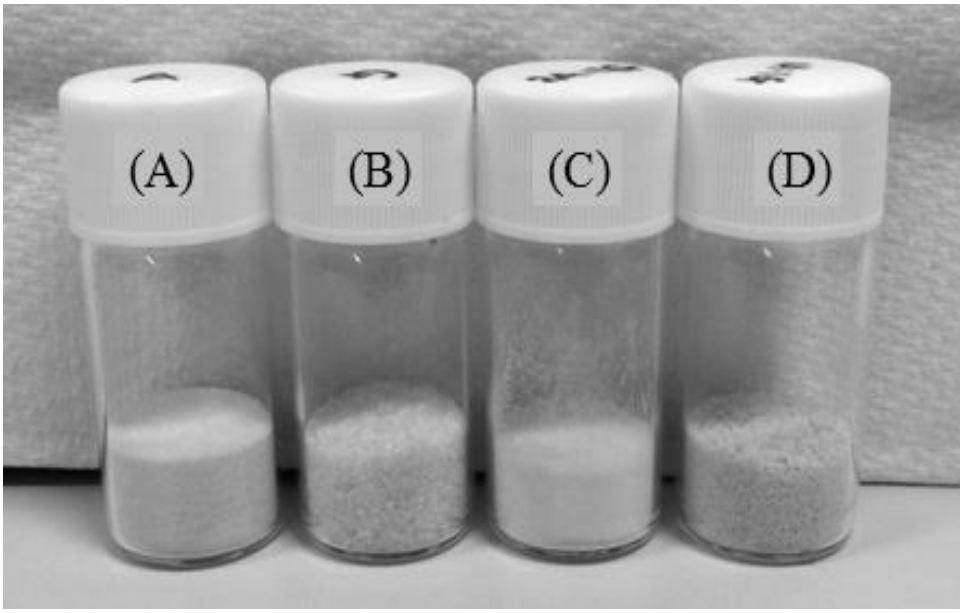


Figure 6.2 Images of (A) agar powder; (B) gelatin powder; (C) berberine; and (D) berberine loaded agar/gelatin microcapsules

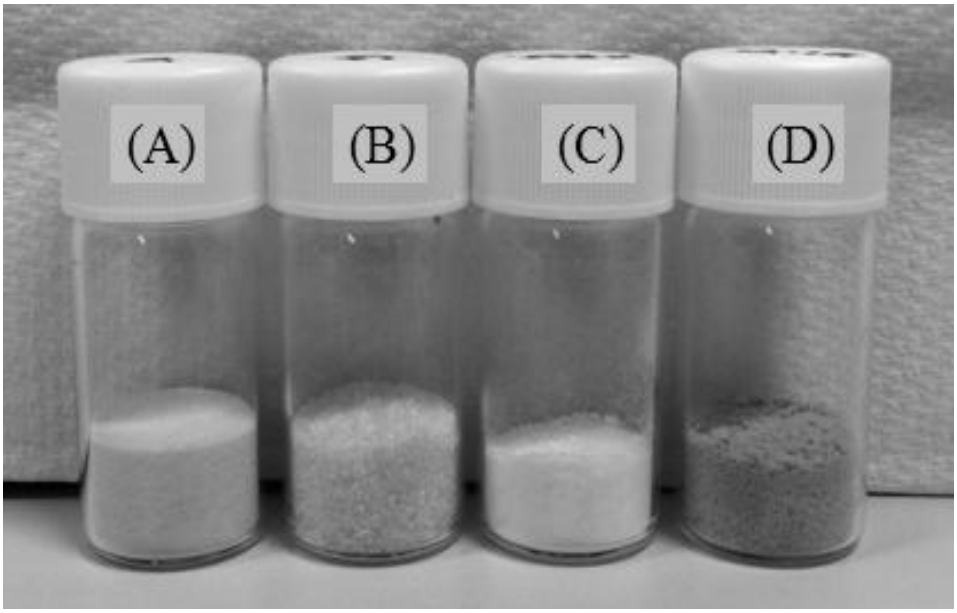


Figure 6.3 Images of (A) agar powder; (B) gelatin powder; (C) gallic acid; and (D) gallic acid loaded agar/gelatin microcapsules

6.2.3 Determination of Encapsulation Efficiency

Drug-loaded microcapsules were dissolved in deionized water by heat. The samples were then filtered. Berberine and gallic acid contents were analyzed by measuring the absorbance at 343nm and 260nm (λ_{max} of drugs in deionized water) after suitable dilution using UV/VIS spectrophotometer (Lambda 18, Perkin Elmer). Each sample was performed in triplicate in order to obtain the mean drug loading efficiency. The drug loading efficiency was calculated as below. Figure 6.4 illustrates the standard calibration curve of berberine and Figure 6.5 shows the standard calibration curve of gallic acid.

$$\text{Drug loading efficiency (\%)} = \frac{\text{Calculated drug concentration}}{\text{Theoretical drug concentration}} \text{ X } 100$$

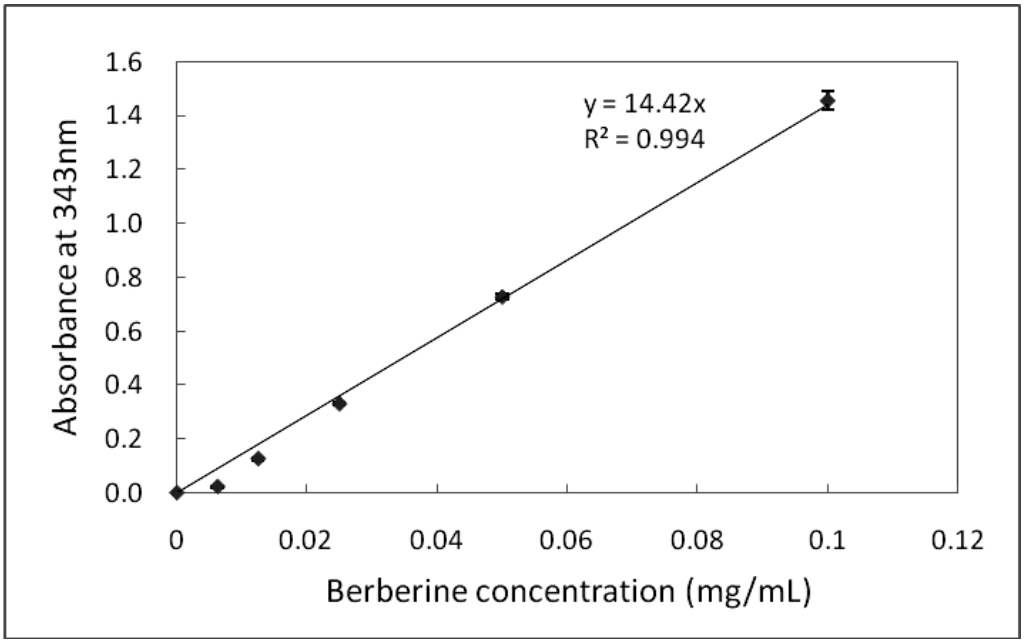


Figure 6.4 Standard calibration curve of berberine

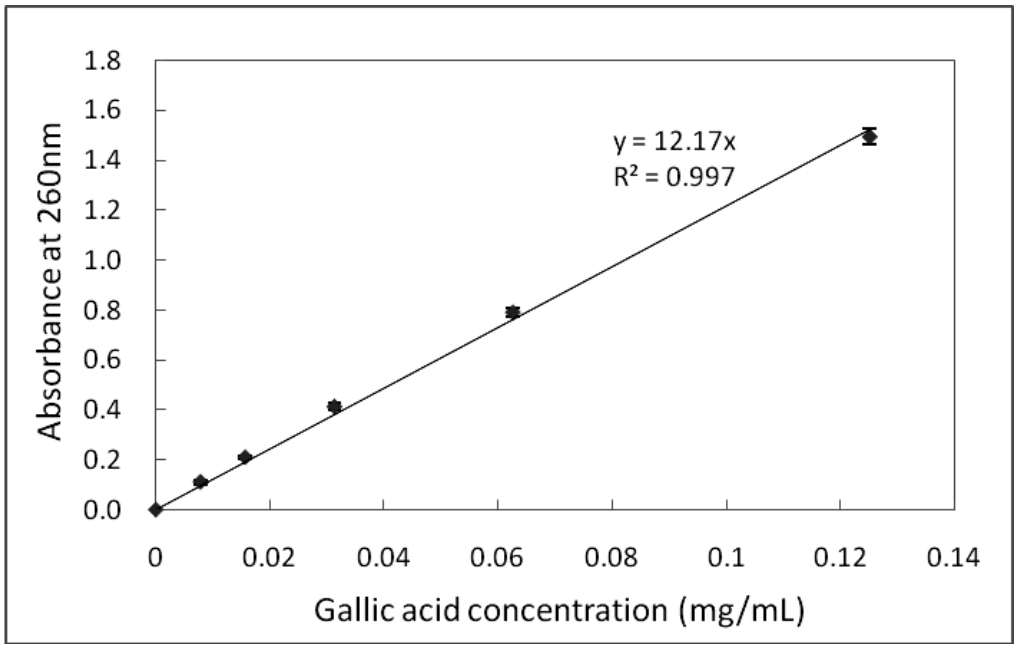


Figure 6.5 Standard calibration curve of gallic acid

6.2.4 Determination of Size of Microcapsules

The particle size of drug-loaded microcapsules was investigated as described in Chapter 3, Section 3.2.4.

6.2.5 Morphological Analysis of Microcapsules

Surface Morphology of drug-loaded microcapsules was evaluated as described in Chapter 3, Section 3.2.5. The SEM was operated at a high magnification up to 2,000X to observe the surface morphology.

6.2.6 FTIR Analysis of Microcapsules

FTIR spectrum of drug-loaded microcapsules was analyzed as described in Chapter 3, Section 3.2.6.

6.2.7 *In Vitro* Controlled Release

For *in vitro* release model of berberine loaded microcapsules, a given amount of microcapsules was firstly suspended in a phosphate solution (pH 7.4) medium for 72 hours and incubated at 100rpm at room temperature. The *in vitro* release model of gallic acid loaded microcapsules was modified from that used by Klinkesorn and McClements (Klinkesorn and McClements, 2009). A given amount of microcapsules was suspended in a primary release medium for 4 hours at 37℃ and incubated at 100rpm. Afterwards, the remaining microcapsules were collected from the primary medium, followed by putting them into a secondary release medium for 8 hours at 37℃ and incubated at 100rpm. The primary and secondary release media consisted of equal volumes of HCl solution pH 1.2 containing 10mg/mL pepsin and 0.2mg/mL lipase (artificial gastric juice); and phosphate solution pH 7.4 containing 10mg/mL trypsin and 3mg/mL lipase (artificial intestine juice) respectively. Aliquots were taken at desired time intervals. The drawn sample was filtered using filter paper and the residue was returned to the suspension medium. Both clear filtrate of the two drug loaded microcapsule samples were analyzed by UV/VIS spectrophotometer (Lambda 18, Perkin Elmer) for the determination of drug content.

6.2.8 Determination of Minimum Inhibitory Concentrations (MICs)

The stock suspension of *Staphylococcus aureus (S. aureus)* was serially diluted 1/100, 1/10,000, 1/1,000,000 and 1/100,000,000 in Luria broth (LB). Ten microlitres of bacteria cells were placed on the agar plates and the plates were incubated at 37˚C for 24 hours. The total number of colonies from each plate was counted manually and the bacteria cell concentration was calculated and adjusted to 1 x 10^6cells/mL. The minimum inhibitory concentrations (MICs) of berberine

and berberine loaded microcapsules was determined by the LB broth dilution method. The diluted *S. aureus* in a concentration of 1 X 10^6cells/mL was added to the 96 wells microtitre plate. Various concentrations of berberine and berberine loaded microcapsules were added from a starting concentration of 500μg/mL and they were diluted serially. Methicillin was used as the positive control while blank microcapsules (without drugs) were used as a negative control. They were then incubated at 37˚C for 24 hours.

6.2.9 Antibacterial Study of Berberine Loaded Microcapsules

S. aureus was used to study the effectiveness of Berberine loaded microcapsules against its grown in culture. *S. aureus* were diluted with broth medium and plated on preset agar plate. Filter papers (3MM, Whatman) were cut in circular shape with 1 cm in diameter. The microcapsules and drugs (both with different concentrations), blank microcapsules without drugs (as negative control) and methicillin (as positive control) on the strip were placed on the surface of the agar. The plates were placed in incubator at 37˚C for 24 hours and growth inhibition of *S. aureus* on the agar plates were recorded according to the scheduled time interval.

6.2.10 Antibacterial Assessment of Berberine Loaded Microcapsules on Cotton Fabrics

The antibacterial assessment was conducted using the parallel streak method with reference to American Association of Textile Chemists and Colorists test method. LB and LB with bacterial agar were prepared in deionized water respectively. They were then sterilized at 121˚C for 20 minutes. Fifteen milliliters of sterilized nutrient agar solution was poured into each standard flat bottomed petri dish. The

LB-agar was allowed to gel firmly before inoculating. *S. aureus* was retrieved from its frozen glycerol stock at -20˚C and streaked on a LB-agar plate in order to obtain single colonies. Afterwards, a single colony was picked up and inoculated in LB at 37˚C for 24 hours. The diluted inoculum was prepared by transferring 1mL of a 24 h broth culture into 9mL of sterile deionized water. The streaked LB-agar plates were performed by using a 4mm inoculating loop to load one loopful of the diluted inoculum and transferring to the surface of the sterile agar plate by making five streaks approximately 60mm in length, spaced 10mm apart covering the central area of a standard petri dish without refilling the loop. The microcapsule and drug (both with different concentrations) fabricated cotton samples were gently placed transversely across the five inoculum streaks to ensure contacting with the LB-agar surface. The plates with test specimens were then incubated at 37 ± 2˚C for 24 hours.

6.2.11 *In Vitro* Berberine Delivery Model Using Nude Mice Skin

Nude mice were sacrificed and autopsy was performed to obtain the skin immediately. They were stored under ice condition in order to keep them fresh. The skin was immersed into the cell culture medium. The microcapsules containing 0.625mg/cm^2 berberine and blank microcapsules without drugs were fabricated on the cotton fabrics respectively. The microcapsule treated cotton samples were fixed on the skin at room temperature for 24 hours. The cotton fabrics were then removed carefully and they were used for further release analysis. The mice skin was washed with phosphate buffer and placed under the UV illumination and the florescent images were captured.

6.2.12 Hepatoprotective Effect of Gallic Acid Loaded Microcapsules

Eight weeks old C57BL/6 mice, weighing approximately 20-25g, were purchased from the animal unit of The Chinese University of Hong Kong and maintained in a conventional sanitary facility, in accordance with the institutional guidelines on animal care, with the required consistent temperature and relative humidity. All the procedures were approved by the Animal Research Ethics Committee. A total of 10 mice were included in our study and they were divided into three groups respectively. The 50mg/kg gallic acid and microcapsules containing 50 mg/kg gallic acid were mixed with the sterilized deionized water respectively. On day 1, 550mg/kg acetaminophen (toxic dose) was intraperitoneally injected to all 10 mice in order to induce the acute hepatic toxicity and damage. From day 2 to day 4, treatment groups received microcapsules once daily at the predetermined concentration (50μg drug/g body weight). Positive control groups received gallic acid once daily at the concentrations of 50μg drug/g body weight. Negative control group received the same volume of sterilized deionized water orally. The animals were monitored and recorded if there had any abnormal behaviors. On day 5, all the mice were sacrificed and autopsies were performed to collect peripheral blood and livers for analytical chemistry and pathological analysis. Sections of mouse liver from autopsy samples were dewaxed with xylene and gradient concentrations of ethanol. Slides were stained with haematoxylin and finally they were inspected under a light microscope to serach for any possible necrotic features. All the blood was collected after the mice were sacrificed and plasma was isolated after centrifugation. Afterwards, plasma enzyme included alanine aminotransferase (ALT) and phosphate (Phos) were measured by the Vet biochemistry assay kits (Hau *et al.*, 2010).

6.3 Results and Discussion

6.3.1 Influence of Agar to Gelatin Ratio

The formation of microcapsules can be affected by the agar to gelatin ratio. In fact, the determination of a suitable ratio between two polymers (agar and gelatin) was very important for the successful encapsulation of the drugs into the matrix of microcapsules. From Figure 6.6, it was observed that the drug loading efficiency of both berberine and gallic acid loaded microcapsules reached the maximum level when the agar to gelatin ratio was 2:1. The drug loading efficiency of drug loaded microcapsules decreased with the increased gelatin ratio. It was also noticed that the particle size of both drug loaded microcapsules generally increased with the increment of the gelatin ratio. Therefore, the agar to gelatin ratio of 2:1 was selected to give better quality of microcapsules (highest drug loading efficiency and satisfactory particle size) and develop the microcapsules for the remaining studies.

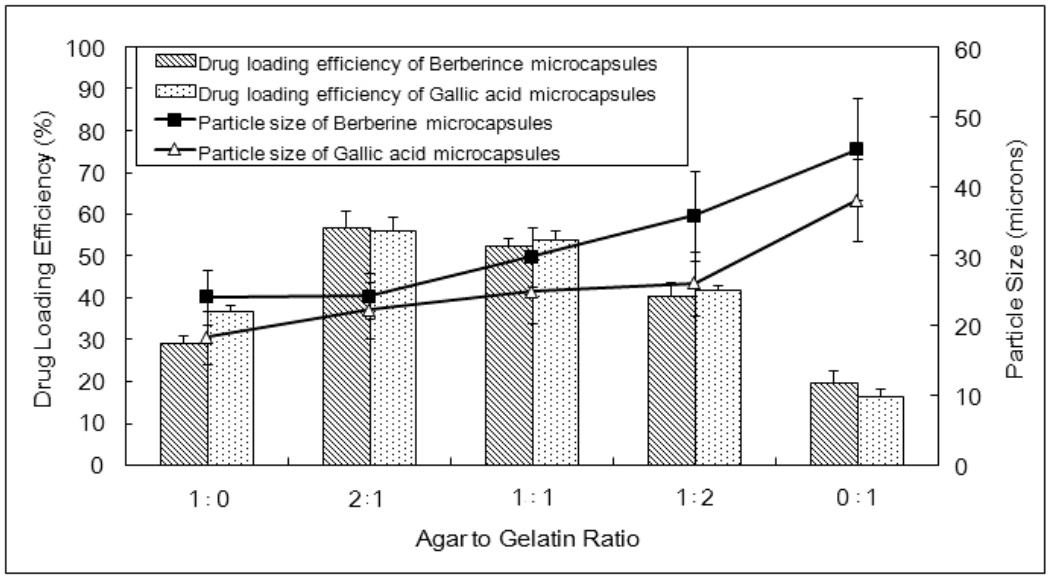


Figure 6.6 Influence of agar to gelatin ratio on the drug loading efficiency and particle size of microcapsules

6.3.2 Influence of Polymer to Oil Ratio

The polymer to oil ratio also affected the drug loading efficiency and particle size of microcapsules. Figure 6.7 shows that both berberine and gallic acid loaded microcapsules reached the highest drug loading efficiency in the polymer to oil ratio of 1:40. It was revealed that in general, both drug loaded microcapsules reduced their particle size as the oil ratio increased. This might be due to the fact that the microparticles were easily aggregated and they were difficult to be formed in the insufficient oil volume, resulting in larger size of developed microcapsules. However, the emulsion was agitated without difficulties during the microcapsule manufacturing process and the microparticles were easily separated and produced. It was hypothesized that more oil volume at the interface tends to break down the W/O emulsion into smaller droplets. According to the result of the drug loading efficiency and particle size of microcapsules, the polymer to oil ratio was chosen as 1:40 so as to obtain the microcapsules with higher drug loading efficiency and acceptable particle size.

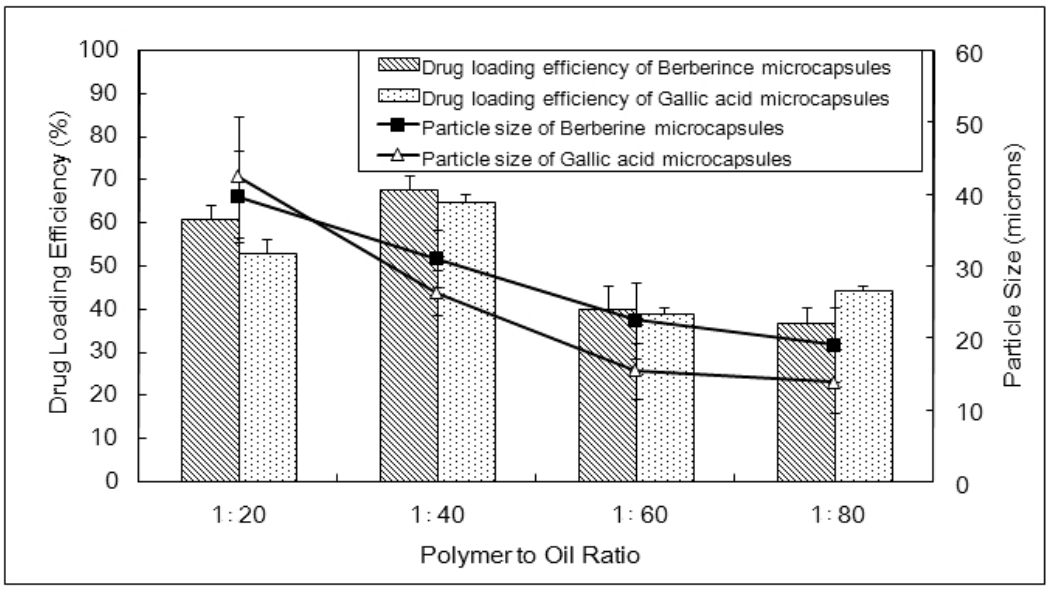


Figure 6.7 Influence of polymer to oil ratio on the drug loading efficiency and particle size of microcapsules

6.3.3 Influence of Surfactant Volume

The surfactant volume applied to microcapsule formation process also had an effect on the drug loading efficiency and particle size of microcapsules. It was shown that the drug loading efficiency of both berberine and gallic acid loaded microcapsules reached the maximum level at 1% (v/v) of the surfactant volume and then dropped down (Figure 6.8). The particle size of drug loaded microcapsules increased with a reduction of the surfactant input in the microencapsulation system. This might be resulted from the fact that more input of surfactant led to the decrease in surface tension in the interface between the water phase and oil phase. This might lead to the better stabilization of emulsified droplets with an increment of surfactant concentration preventing coalescence and aggregation of small droplets during the microcapsule production. Therefore, the surfactant volume was selected to be 1%v/v in order to form the microcapsules with highest drug loading efficiency and satisfactory particle size.

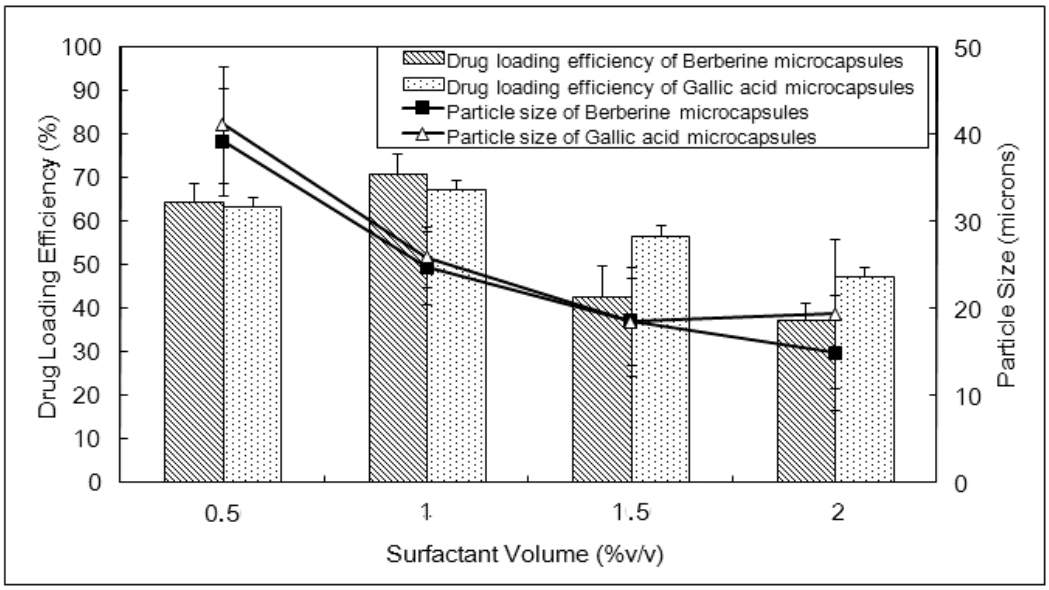


Figure 6.8 Influence of surfactant volume on the drug loading efficiency and particle size of microcapsules

6.3.4 Influence of Stirring Speed

The stirring speed introduced to the microcapsule formation could also affect the drug loading efficiency and particle size of microcapsules. As shown in Figure 6.9, the drug loading efficiency of both berberine and gallic acid loaded microcapsules increased from the stirring speed of 600 rpm and met the highest level at 1000 rpm. It was illustrated that the particle size of both drug loaded microcapsules generally decreased when the stirring speed increased. This might be associated to the fact that a stronger stirring speed possibly breaks the W/O emulsion into smaller particle droplets and prevents the aggregates of smaller microcapsule forming during the microcapsule formation stage. On the contrary, slower stirring speed could induce to agglomeration of smaller microparticles which aggregates to become larger particles. The stirring speed of 1000 rpm, therefore, was selected in order to produce the microcapsules with desirable drug loading efficiency and suitable particle size.

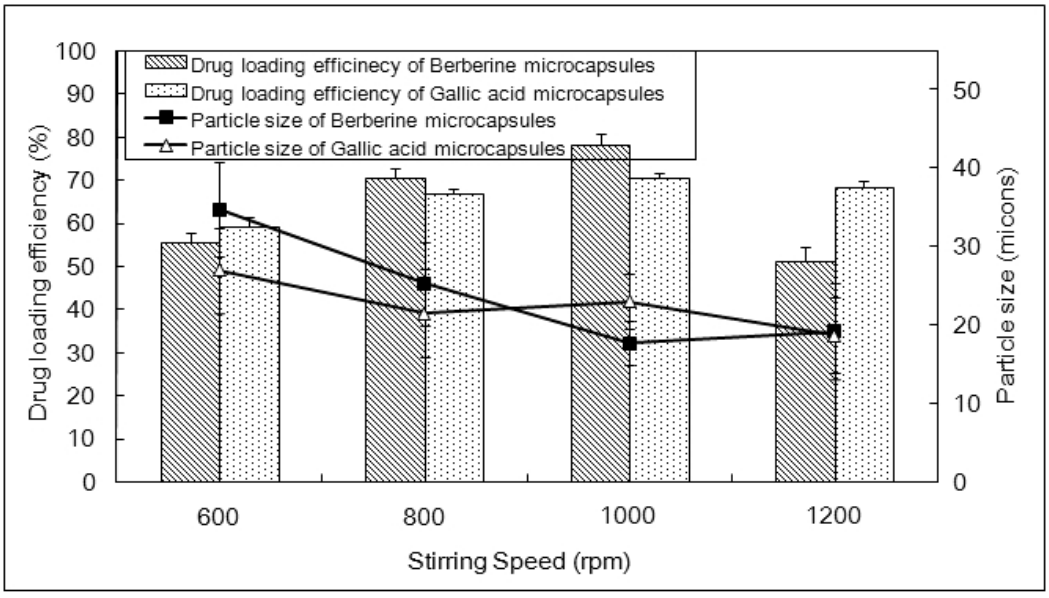


Figure 6.9 Influence of stirring speed on the drug loading efficiency and particle size of microcapsules

6.3.5 Morphological Analysis of Microcapsules

The data shown in Figure 6.10 and 6.11 demonstrate that both berberine and gallic acid loaded microcapsules were approximately spherical in shape with certain level of agglomerations. The particle sizes of the optimised berberine and gallic acid loaded microcapsules were in the range of 7.14 to 21.43μm and 16.11 to 27.22μm respectively.

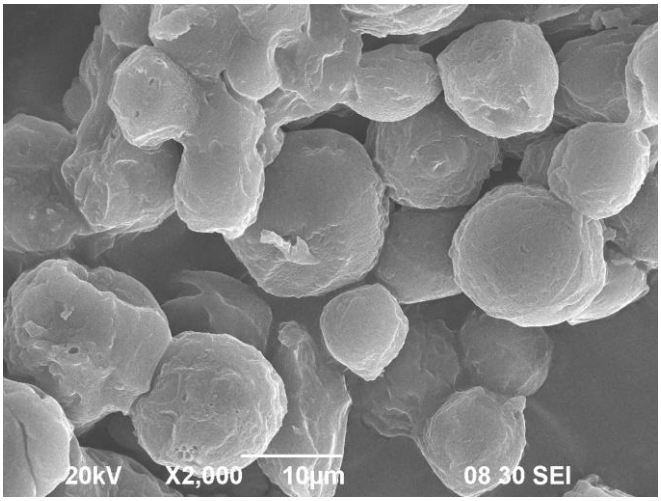


Figure 6.10 SEM image of berberine loaded microcapsules

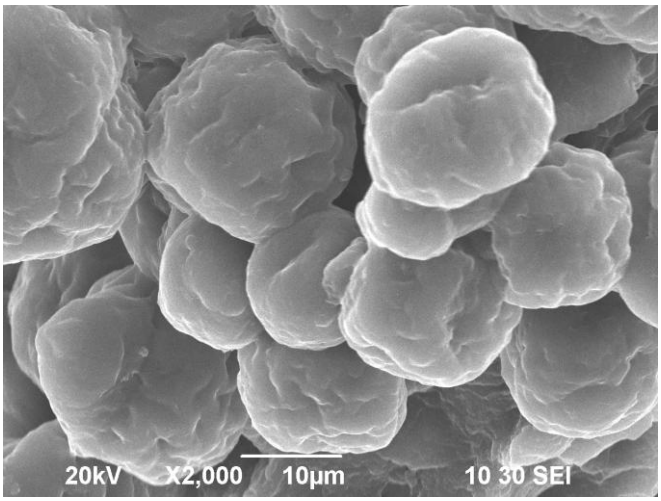


Figure 6.11 SEM images of gallic acid loaded microcapsules

6.3.6 FTIR Analysis

In this study, the diffuse reflectance mode measurement was applied in the range of wavelengths from 4000 to 700cm^{-1} in wave-numbers. The infrared absorption spectra of berberine and gallic acid loaded agar/gelatin microcapsules are shown in Figure 6.12 and 6.13. Significant peaks were observed in agar spectrum at 2926cm^{-1}, 1657cm^{-1} and 1383cm^{-1} that were related to C-H stretching, C-C stretching and the C-H vibration respectively. Gelatin contained the characteristic amide absorption bands at 1650cm^{-1} and 1542cm^{-1} which referred to N-H stretching. Berberine exhibited the significant peaks at 2920cm^{-1} and 2850cm^{-1} that represented the C-H stretching (alkanes), 1505cm^{-1} (aromatic C=C vibrations), 1103cm^{-1} (ring deformation and CH in-plane bending) and 1035cm^{-1} (C-H vibrations). The characteristic peaks for gallic acid at 3492cm^{-1}, 3370cm^{-1} and 3282cm^{-1} corresponded to different modes of O-H groups. The bands in 2920cm^{-1} and 2850cm^{-1} region were assigned to aromatic C-H stretching. Gallic acid also contained the characteristic absorption at 1701cm^{-1} (carboxylic acids), 1615cm^{-1} (C=C stretching) and 1265cm^{-1} (C=O stretching).

The spectrum of berberine containing microcapsules (Figure 6.12) showed the characteristic peaks of berberine, agar and gelatin. The peak at 1653cm^{-1} detected the presence of C-C stretching from agar and N-H stretching from gelatin in the developed microcapsules. The absorbance at λ = 1545cm^{-1} occurred in microcapsule spectrum showed the N-H stretching from gelatin while the peaks at 1383cm^{-1} demonstrated the C-H vibration from agar. The absorbance at 2922cm^{-1} and 2922cm^{-1} revealed the existence of C-H stretching from berberine. The FTIR spectrum of the microcapsule also showed the absorption of berberine slightly shifted to 1506cm^{-1} (aromatic C=C vibrations), 1100cm^{-1} (CH in-plane

bending) and 1036cm^{-1} (C-H vibrations). Based on the FTIR results, it was noticed that the developed microcapsules contained the chemical ingredients and functional groups of the wall material (agar and gelatin) and the core active ingredient (berberine). The overall results confirm the synthesis of berberine loaded microcapsules.

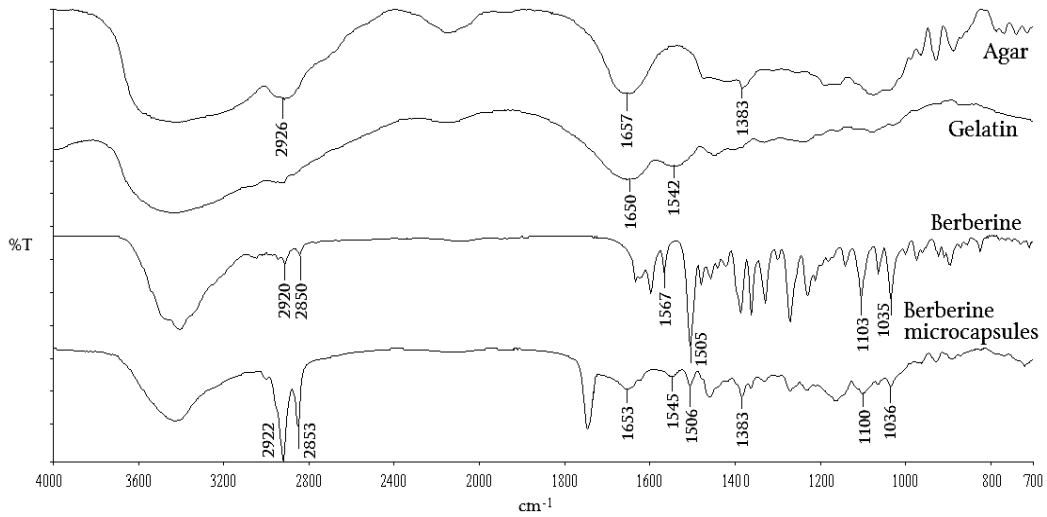


Figure 6.12 FTIR spectrum of berberine loaded agar/gelatin microcapsules

The spectrum of gallic acid loaded microcapsules (Figure 6.13) showed the characteristic peaks of gallic acid, agar and gelatin. The peak at 1655cm^{-1} revealed the existence of C-C stretching from agar and N-H stretching from gelatin in the developed microcapsules. The absorbance at 1383cm^{-1} detected the C-H vibration from agar. The characteristic peaks for gallic acid slightly shifted to 3493cm^{-1}, 3395cm^{-1} and 3283cm^{-1} representing different modes of O-H groups occurred in the microcapsule spectrum. The peaks at 2920cm^{-1} and 2850cm^{-1} demonstrated the presence of aromatic C-H stretching from gallic acid. The FTIR spectrum of the microcapsule also exhibited the characteristic peaks of gallic acid at 1701cm^{-1} (carboxylic acids), 1615cm^{-1} (C=C stretching) and 1265cm^{-1}

(C=O stretching). Based on the FTIR results, it was proven that the microcapsules contained the chemical ingredients and functional groups of the wall material (agar and gelatin) and the core active agent (gallic acid). The overall results confirm the synthesis of gallic acid loaded microcapsules.

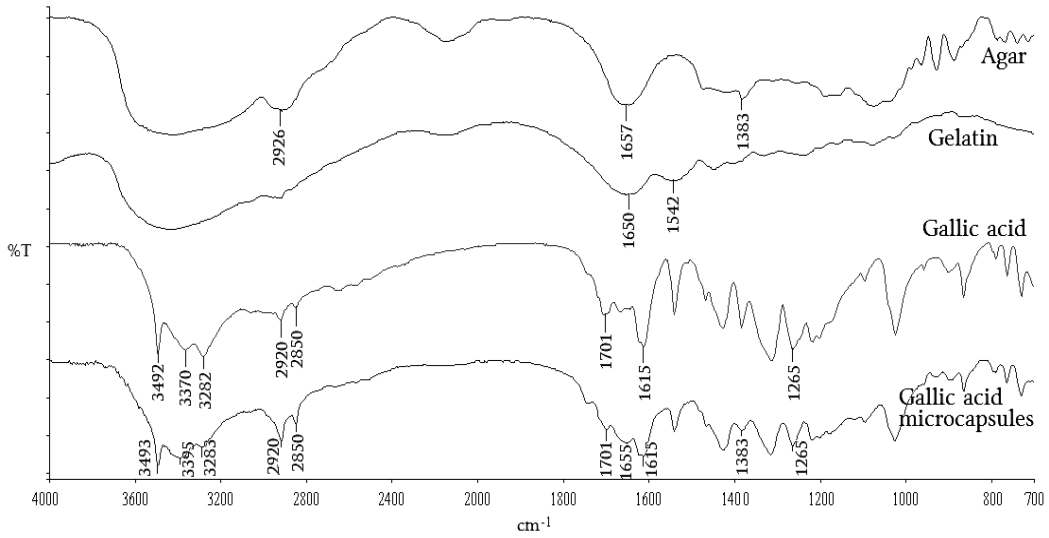


Figure 6.13 FTIR spectrum of gallic acid loaded agar/gelatin microcapsules

6.3.7 *In Vitro* Controlled Release

In the *in vitro* controlled release model for studying berberine loaded microcapsules, the percentage of drug release was investigated for 72 hours and the results obtained are shown in Figure 6.14. The drug was released gradually in the phosphate (pH 7.4) medium. After the first 6 hours, it was observed that approximately 40% of berberine was released from microcapsules. After 24 hours, another 10% of the drug was released, where 48 and 72 hours were closed to the plateau level.

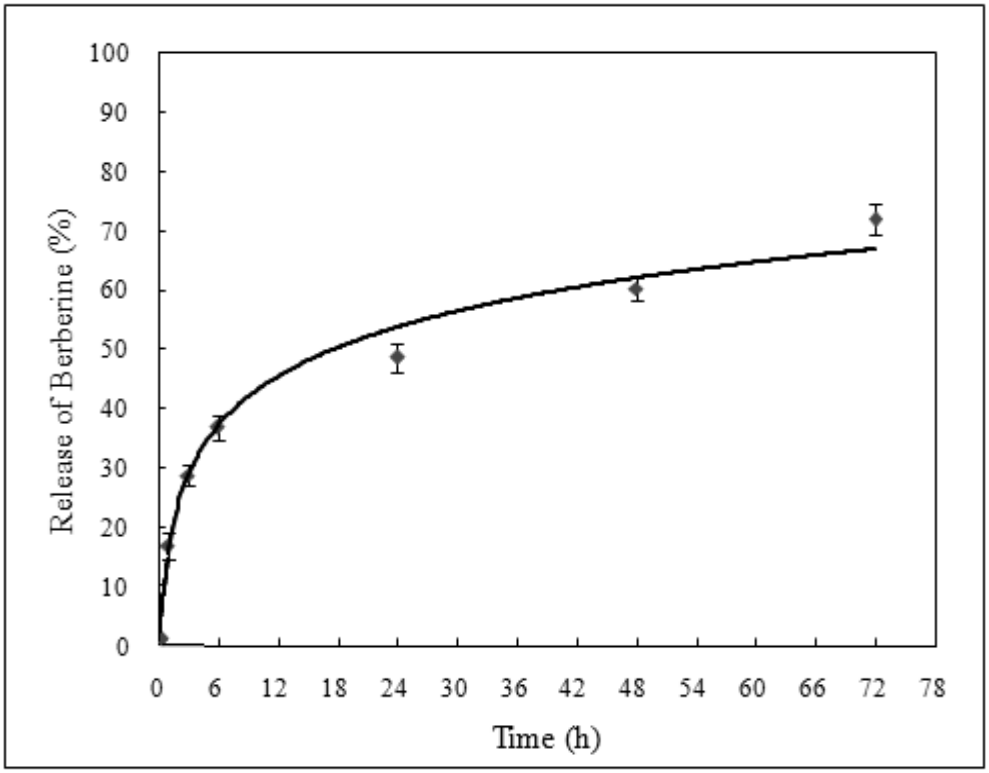


Figure 6.14 Release of berberine loaded microcapsules

In the release model of gallic acid loaded microcapsules, the percentage of drug release was monitored for 12 hours under two release mediums and shown in Figure 6.15. Gallic acid was more readily released from microcapsules when subjected to the primary release medium of HCl (pH 1.2) which simulated the stomach condition. It was found that more than 60% of the drug was released in the first 4 hours in HCl medium simulating the stomach condition in human body. This may be probably due to the fact that the agar-gelatin wall matrix of microcapsules was more susceptible to the acidic condition as there were some micropores on the surface of microcapsules, resulting in higher leakages of core drugs. Gallic acid was continuously released at a slower speed in the secondary medium of phosphate (pH 7) which simulated the intestinal condition as agar-gelatin wall matrix of microcapsules was more stable and became more

rigid in alkaline condition, and approximately 90% of the drug was released after 12 hours.

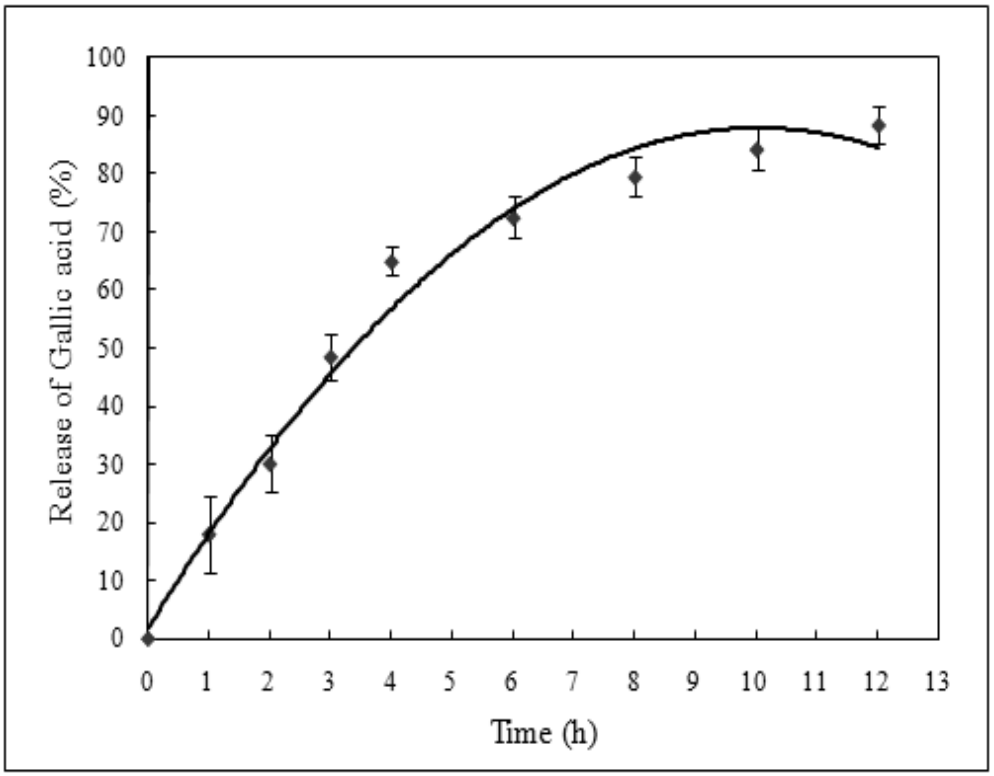


Figure 6.15 Release of gallic acid loaded microcapsules

6.3.8 Determination of Minimum Inhibitory Concentrations (MICs)

The MIC represented the lowest concentration of berberine allowing no growth of tested bacteria as shown in Table 6.1. Methicillin was used as a positive control in the MICs study. It was observed that the MIC for *S. aureus* of berberine containing microcapsules was 47μg/mL while that of berberine was 94μg/mL. The MICs revealed that berberine loaded microcapsules was found to be more active against *S. aureus* when compared with the free drug. The more effective growth inhibition of berberine loaded microcapsules might be due to the controlled release of the microcapsules. The sustained release of drugs from

microcapsules enabled the extended growth inhibition towards *S. aureus*.

Table 6.1 Minimum inhibitory concentration (MIC) of berberine loaded microcapsules and berberine

	MIC (μg/mL)			
	Methicillin	Berberine	Berberine loaded microcapsules	Blank microcapsules
S. aureus	3	94	47	> 500

6.3.9 Antibacterial Study of Berberine Loaded Microcapsules

Figure 6.16 shows that the berberine loaded microcapsules remarkably improved the anti-*S. aureus* activity when compared to that of berberine. The zone of clearance of both berberine loaded microcapsules and berberine was shown in Table 6.2. A dose response relationship could be observed. It was found that higher concentration of berberine in microcapsules exhibited better antibacterial activity towards *S. aureus*. However, there was no any significant growth inhibition of berberine towards *S. aureus*. This might be associated with the fact that the berberine was gradually released under control from microcapsules, providing a longer lasting inhibition of bacteria growth. The free berberine was, on the contrary, released at one time and inhibited only the initial growth of bacterium. The result revealed the outstanding improvement of berberine containing microcapsules as compared to the free drug form.

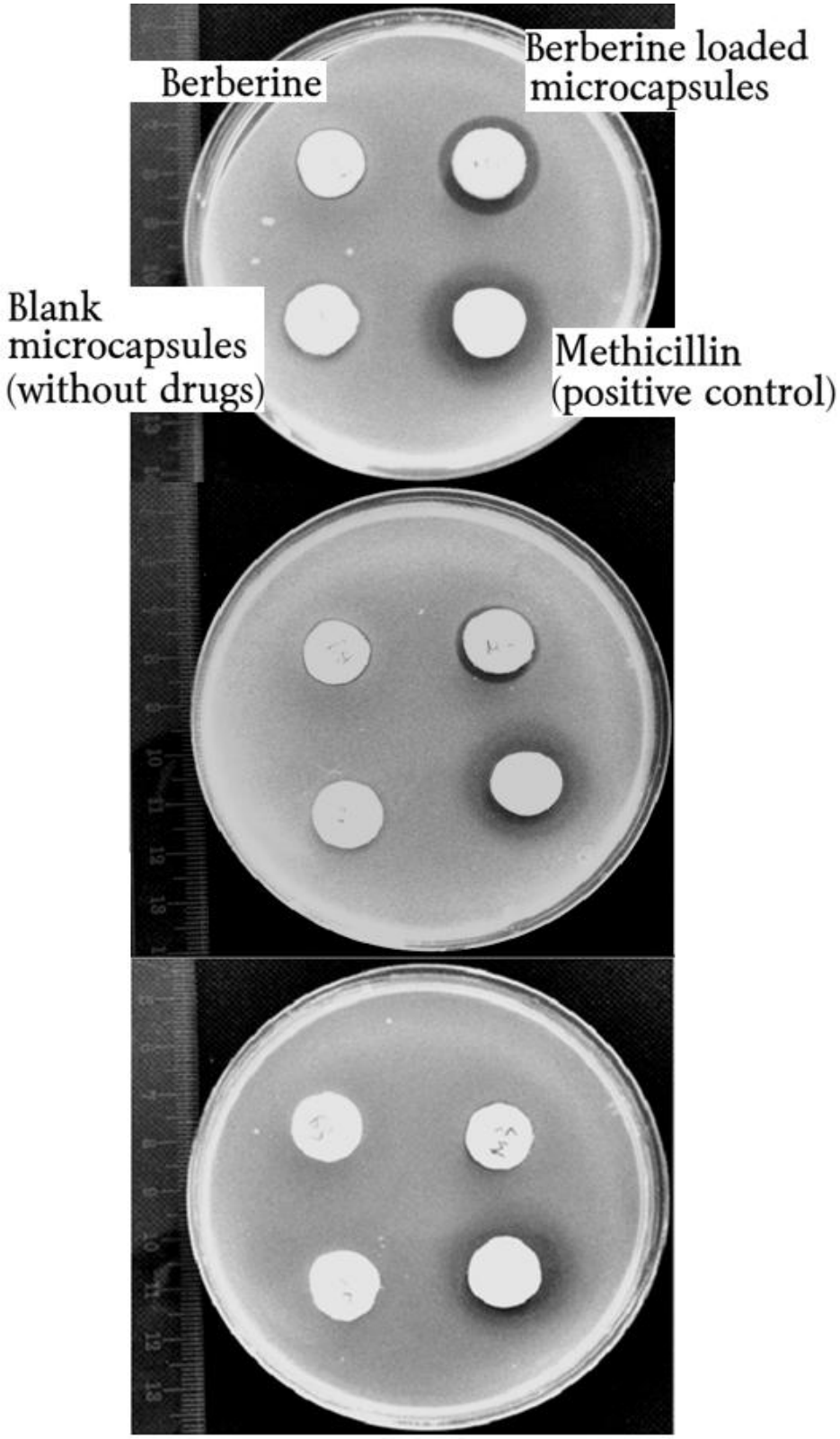


Figure 6.16 Growth inhibition of *S. aureus* after 24hours. The berberine concentration (from upper to lower column): 1.25mg/cm^2, 0.625mg/cm^2 and 0.3125mg/cm^2

Table 6.2 Zones of clearance (mm) of berberine loaded microcapsules and berberine

	Zones of clearance (mm)		
Berberine concentration	Berberine loaded microcapsules	Berberine	Blank microcapsules (Negative control)
0.3125mg/cm²	-	-	-
0.625mg/cm²	1.93 ± 0.01	0.40 ± 0.01	-
1.25mg/cm²	2.83 ± 0.03	1.07 ± 0.01	-

Methicillin was used as the positive control and its clear zone (mm) was 5 ± 0.05.

6.3.10 Antibacterial Assessment of Berberine Loaded Microcapsules on Cotton Fabrics

Figure 6.17 demonstrated that the berberine loaded microcapsules on cotton fabrics significantly improved the anti-bacterial activity towards *S. aureus* when compared with the free drug. Table 6.3 showed the zones of clearance of both berberine loaded microcapsules and berberine. A dose response relationship could be found. These results were consistent with those of above antibacterial study. The results suggested that the Berberine loaded microcapsules could be applied to textile materials to promote the anti-*S. aureus* property.

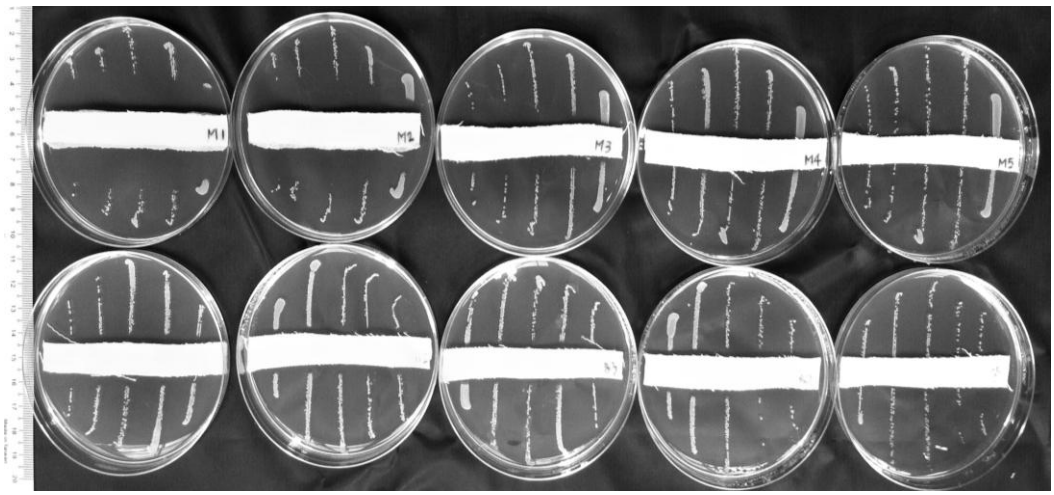


Figure 6.17 Growth inhibition of *S. aureus* after 24hours. The upper row represents the berberine loaded microcapsules on cotton fabrics and the lower row represents the berberine on cotton fabrics (from left to right column: 1.25mg/cm^2, 0.625mg/cm^2, 0.3125mg/cm^2, 0.15625mg/cm^2, zero-control)

Table 6.3 Zones of clearance (mm) of berberine loaded microcapsules and berberine on cotton fabrics

	Zones of clearance (mm)		
Berberine concentration	Berberine loaded microcapsules	Berberine	Blank microcapsules (Negative control)
0.15625mg/cm²	1.07 ± 1.00	1.30 ± 0.67	-
0.3125mg/cm²	4.05 ± 2.38	1.50 ± 0.61	-
0.625mg/cm²	11.15 ± 2.75	1.85 ± 0.29	-
1.25mg/cm²	13.05 ± 1.96	2.75 ± 0.47	-

6.3.11 Analysis of *In Vitro* Berberine Delivery Model Using Nude Mice Skin

Figure 6.18 shows that the berberine of microcapsule fabricated cotton samples were delivered to the nude mice skin. This was further demonstrated using the UV illuminant; and the fluorescence could be observed. The mean release of berberine from microcapsule treated cotton fabrics was 41.08% ± 7.68. This result was consistent with the *in vitro* release model.

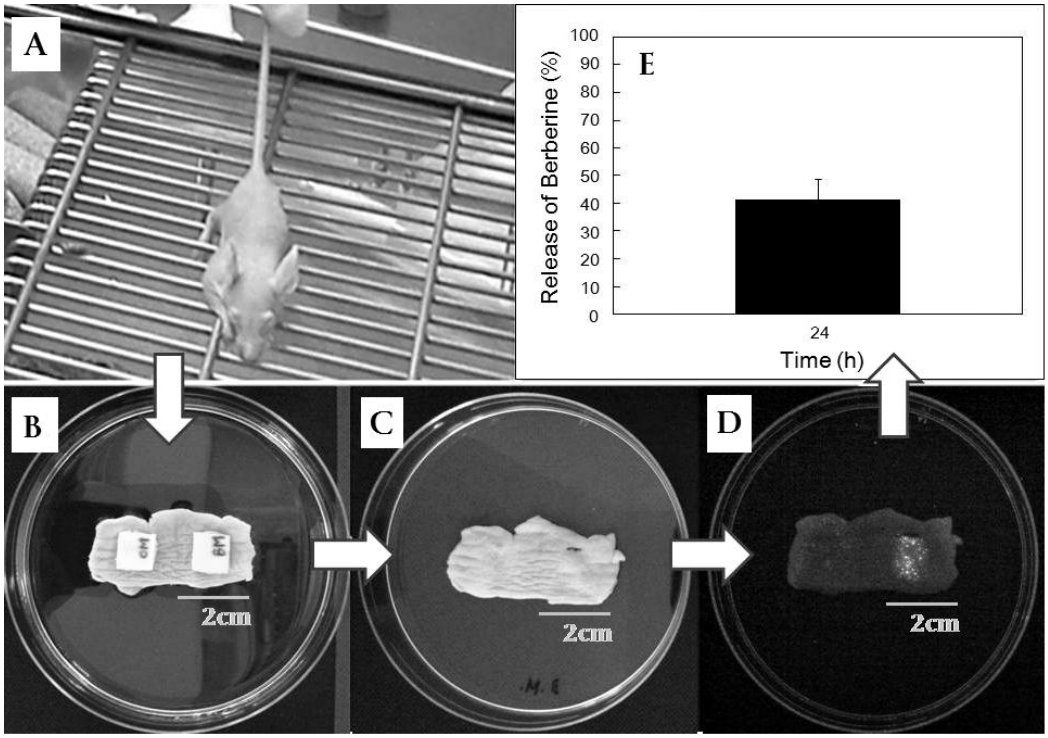


Figure 6.18 Nude mice skin experiment was performed to demonstrate the possible delivery of berberine from microcapsule fabricated cotton: (A) nude mouse; (B) nude mouse skin treated with microcapsule fabricated cotton samples; (C) nude mouse skin treated with microcapsule fabricated cotton after 24h; and (D) nude mouse skin under UV illumination and E. release of berberine from microcapsule fabricated cotton after 24h. For (B), (C), and (D), the left sample represents the microcapsule without drugs and the right

sample represents the berberine loaded microcapsules. The data in panel (E) represent the mean from *in vitro* release of berberine loaded microcapsule experiments ± SD from three independent experiments

6.3.12 Hepatoprotective Effect of Gallic Acid Loaded Microcapsules

Overdose of acetaminophen (APAP) is one of the common types of drug poisoning which can cause fatal damage of vital organs, such as liver, kidneys and heart. Gallic acid loaded microcapsules administrated at 50mg/kg gallic acid were found to be helpful in APAP overdose-treated mice if the treatment was delayed to 24 hours. Although 75% of APAP overdose-treated mice still survived after 4 days (Table 6.4), all the mice showed the necrotic liver feature (Figure 6.19) and 2 of the 3 mice exhibited the kidney necrosis. For the microcapsule treated group, all the mice still survived after the whole treatment period. Liver and kidney autopsies revealed cytoplasm integrity of the hepatocytes in 2 of the 3 mice from the microcapsule group (Figure 6.20). Only 1 of them exhibited a certain degree of liver and kidney damage. However, 1 mouse in the gallic acid treated group survived after 4 days. It was observed that all the liver and kidney autoptic sections demonstrated the necrotic phenomenon after the treatment.

Table 6.4 Survival percentage of mice treated with APAP alone (on day 1), gallic acid loaded microcapsules (single dose daily from day 2–4), and gallic acid (single dose daily from day 2–4)

Study group	Mice, n	Day 1	Day 2	Day 3	Day 4
Water control group	4	100	100	100	75
APAP group (50mg/kg Gallic acid loaded microcapsules)	3	100	100	100	100
APAP group (50mg/kg Gallic acid)	3	100	100	66.7	33.3

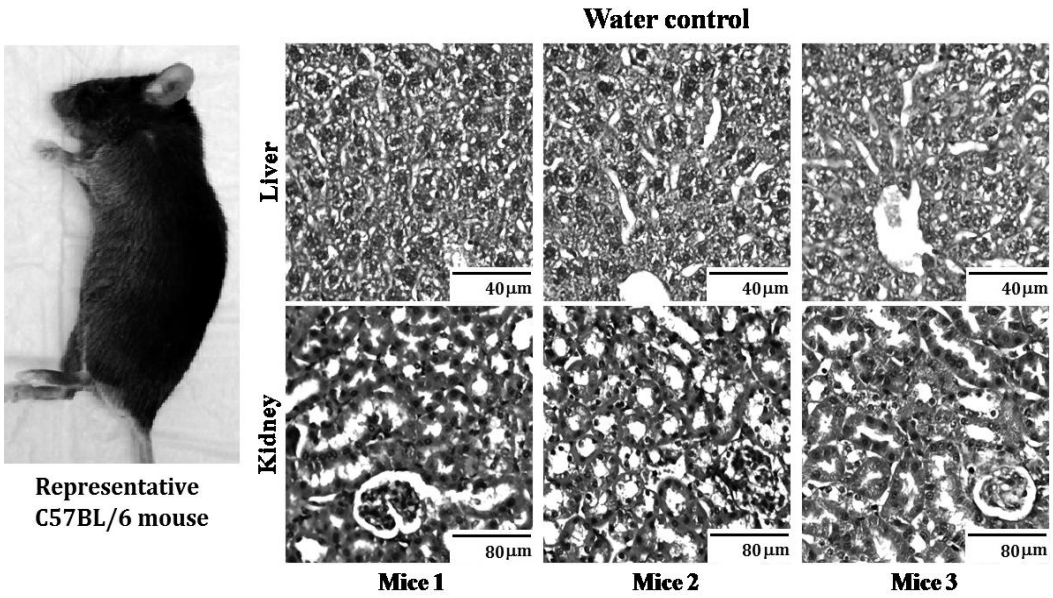


Figure 6.19 H and E histochemical analysis of liver and kidney sections from 3 survived mice treated with APAP alone (on day 1) and deionized water (single dose daily from day 2–4). It should be underlined that all the liver sections exhibit necrosis whereas 2 of 3 of the kidney sections exhibit necrosis and only mice 1 showed a certain degree of cellular integrity in its kidney section

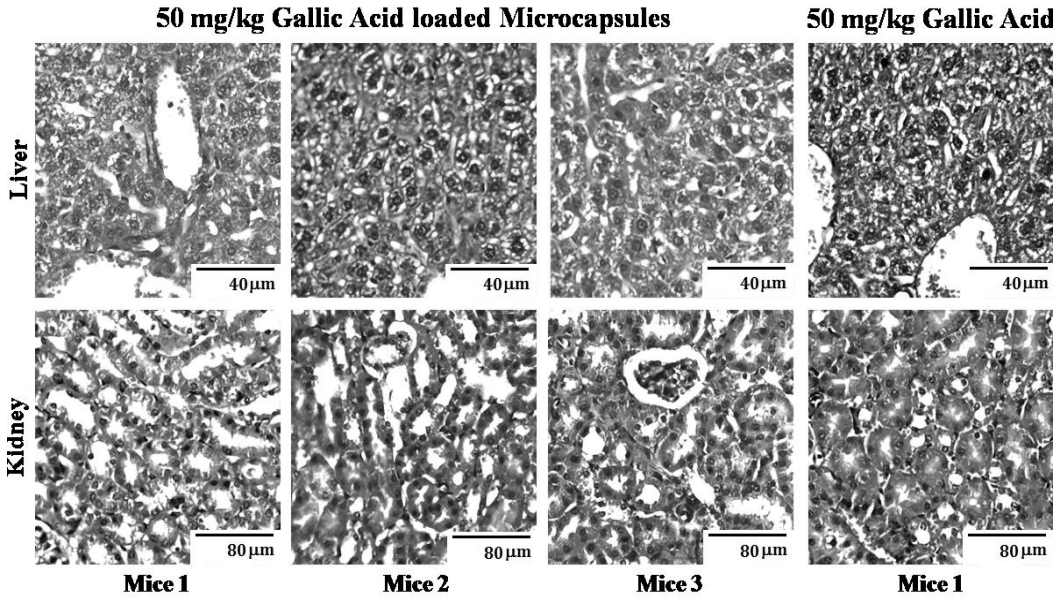


Figure 6.20 H and E histochemical analysis of liver and kidney sections from 3 survived mice treated with APAP alone (on day 1) followed by gallic acid loaded microcapsules (single dose daily from day 2–4), and a survival mouse treated with APAP alone (on day 1) followed by gallic acid (single dose daily from day 2–4). It should be underlined that 1 of 3 of the liver and kidney sections (mice 2) from microcapsule treated group exhibit necrosis while those of gallic acid treated mouse exhibited necrosis

6.3.13 Plasma Liver and Kidney Functional Enzymes Test

When certain types of cells are injured, they may leak some enzymes into the blood. Alanine aminotransferase (ALT) is one of the specific enzymes that predominates in the liver. Therefore, if the liver is damaged, ALT level in blood plasma will increase. Phosphate (Phos) is a charged particle containing the mineral phosphorus. The kidney helps to control the amount of Phos in blood. Additional Phos is filtered by the kidney and excreted in urine. A high level of Phos in blood may lead to a kidney problem.

It was found that ALT level and Phos level in 50 mg/kg gallic acid loaded microcapsule treated group was noticeably lower than that of the water control and gallic acid treated groups (Table 6.5). We speculated that the sustained release of gallic acid from microcapsules might offer a better therapeutic advantage than that of free gallic acid against the damage on both liver and kidney after an overdose administration of APAP.

Table 6.5 Plasma liver and kidney functional assays from mice treated with APAP alone (on day 1), gallic acid loaded microcapsules (single dose daily from day 2–4), and gallic acid (single dose daily from day 2–4)

	Water control	APAP (50mg/kg Gallic acid loaded microcapsules)	APAP (50mg/kg Gallic acid)
ALT (U/L)	34.3 ± 6.1	27 ± 5.3	48
Phos (mg/dL)	8 ± 0.4	7.2 ± 0.2	8.6

6.4 Conclusion

This study reported the development, characterization and safety uses of agar/gelatin microcapsules. Generally speaking, formaldehyde is commonly applied as a crosslinker for gelatin microcapsulation in many previous studies. Complex coacervation method has been used to develop the gelatin-acacia microcapsules for encapsulating vitamin A palmitate; and the influence of formaldehyde volume involved in microcapsule formation on the properties of microcapsules was also investigated (Junyapraset *et al.*, 2001). The formaldehyde crosslinked gelatin microcapsules containing vitamin C have been produced using an emulsion hardening technigue (Cheng *et al.*, 2009). Formaldehyde is important to strengthen the wall matrix of gelatin-based

microcapsules through the crosslinking process. However, it is considered harmful to the human body. Therefore, the present study aimed at developing a formaldehyde free approach by employing agar together with gelatin as the wall matrix materials of microcapsules in order to offer an alternative way for microcapsule formation. It was proposed that the drug was distributed into the agar/gelatin wall matrix of microcapsules for sustained release. The developed microencapsulation recipe without introducing formaldehyde could produce the desired properties of microcapsules due to its safety and cost-efficient benefits in microcapsule formation.

Both oral and topical applications were tested using the berberine and gallic acid loaded microcapsules respectively. Microcapsules containing both drugs were prepared combining the optimal parameters identified. The mean drug loading efficiency and the mean particle size of berberine loaded microcapsules were 78.16% and 16.75μm respectively while those of gallic acid loaded microcapsules were 70.28% and 21.98μm respectively. The FTIR analysis confirmed the encapsulation of berberine and gallic acid in microcapsules. Approximately 70% of the drug was released from berberine loaded microcapsules after 72 hours in the *in vitro* skin model while approximately 90% of the drug was released from gallic acid loaded microcapsules after 12 hours in the simulated digestion model. The MICs and antibacterial tests also proved that berberine loaded microcapsules exhibited a better growth inhibition activity towards *S. aureus* when compared with the original drug. *In vitro* drug delivery model also demonstrated the delivery of berberine from microcapsule treated textiles on nude mice skin. The *in vivo* mice disease model also revealed that gallic acid loaded microcapsules were helpful in the treatment of acute liver and

kidney toxicity. The development of agar/gelatin microcapsules was demonstrated to be an efficienct and safe deliverable tool for both oral and topical applications.

CHAPTER 7

EFFECTS OF MULTIPLE WASHING ON COTTON FABRICS CONTAINING BERBERINE MICROCAPSULES WITH ANTI-STAPHYLOCOCCUS AUREUS ACTIVITY

7.1 Introduction

There has been a growing interest in antibacterial finishing associated with textile clothing in order to promote the antibacterial properties to human body in wearing clothes (Yuan and Robin, 2008). Antibacterial textiles are developed to transfer the antibacterial agent to human body via close contact with the skin. It is believed that such textiles are capable of accomplishing the therapeutic purposes to heal the infection induced skin diseases and offer the feeling of well-being. Transdermal drug delivery systems may offer a high possibility to cure the skin infectious aliments when compared to conventional dosage routes. It is able to minimize the risk and inconvenience during the intravenous and oral administrations; prolong the drug effect at a lower dosage level and provide an easier drug administration resulting in better patient compliance (Elliott, 2003; Krousel-Wood *et al.*, 2005; Ma *et al.*, 2009). As a result, many attempts have been put on exploring the transdermal drug delievey systems. Imparting microencapsulated medicines into textile materials is one of the ways to offer the topical treatments to specific skin ailments.

Over several centuries, medicinal plants are considered useful and helpful to treat the human ailments and diseases. The bioactive substances originated from these plants exhibit the potential health advantages to human beings. There are numerous endeavors on an exploration of antimicrobial agents. It was desired if the microencapsulated antimicrobial agents are natural, non-toxic and safe

towards human body. Berberine is a quaternary alkaloid isolated from natural plants originated from Berberidaceae, Coptis, Fumariaceae, Mahonia, Papaveraceae and other herb species that are traditionally used in Chinese herbal medicines for the treatment of the infectious diseases for many years (Cernakova and Kostalova, 2002; Jin *et al.*, 2010; Zhang *et al.*, 2011). Berberine has also been proven to have anti-inflammatory (Choi *et al.*, 2006) and antibacterial (Cernakova and Kostalova, 2002; Jin *et al.*, 2010; Zhang *et al.*, 2011) properties. Berberine can damage the bacterial cell surface structure and inhibit the growth of bacteria (Jin *et al.*, 2010). Berberine has also been used as a natural source for dyeing cellulosic fibres to promote the antimicrobial activity (Kim and Son, 2005).

The therapeutic efficiency and topical performance of drug containing microcapsules varied when the drugs existed in an internal oil phase or an internal aqueous phase within the wall shell or wall matrix of microcapsules. In the study, berberine was employed as a model agent to be encapsulated into both chitosan-based (oil in water) and agar-gelatin (water-in-oil) microcapsules to demonstrate the antibacterial activity of these two microencapsulation systems after various washing cycles. The SEM images, FTIR analysis and anti-*Staphylococcus aureus* (*S. aureus*) activity of the washed samples were evaluated.

7.2 Experimental

7.2.1 Materials

One hundred percent of semi-bleached 3/1 twill cotton fabrics was purchased from the Tai Tung Piece Goods Co., Ltd, Hong Kong. An acrylic binder (Sigma-Aldrich, Germany) was used to enhance the fixation of berberine loaded microcapsules onto the cotton fabric. AATCC 1993 standard reference detergent was obtained from SDL Atlas Ltd, Hong Kong. Agar and Luria both (LB) were bought from Sigma-Aldrich, Germany. All other reagents were supplied by Sigma-Aldrich, Germany.

7.2.2 Preparation of Microcapsules

Berberine containing chitosan microcapsules (BER/CS microcapsules) and berberine containing agar/gelatin microcapsules (BER/AG microcapsules) were prepared as described in Chapter 3, Section 3.2.2 and Chapter 6, Section 6.2.2 respectively.

7.2.3 Preparation of Microcapsule-Treated Cotton Fabrics

One hundred percent of cotton fabrics were washed in 2% of non-ionic detergent at 40^{o}C for 15 minutes using a washing machine (FONG'S-TP-25, Hong Kong). They were then rinsed with deionised water for 5 minutes in order to remove the impurities, greases and other chemical finishing that may scatter on the fabric surface during the manufacturing processes. Subsequently, the cleaned samples were water-extracted for 5 minutes using a hydroextractor (Electrolus-C260R, Tübingen, Germany). The samples were finally dried at 40^{o}C for 45 minutes by a drying machine (Electrolus-T4350, Tübingen, Germany). The dried fabrics were stored under the standard condition at the relative humidity of 65 ± 2% and 21 ±

1 ^{o}C for 24 hours prior to all experiments.

The cotton fabrics were cut into many pieces of 20cm × 20cm. BER/CS microcapsules and BER/AG microcapsules were firstly diluted with deionized water at the liquor ratio of 1:9 respectively. The microcapsule-based solution was stirred for 15 minutes using a magnetic stir plate at a speed of 1200rpm to obtain a better dispersion of microcapsules. An acrylic binder was prepared with the concentration of 0.5%w/v within the total volume of the microcapsule-based solution. The microcapsules containing 0.3125mg of berberine were then sprayed on each cm^{2} of cotton fabric using a spray gun, followed by drying it at room temperature.

7.2.4 Durability of Microcapsule-Treated Cotton Fabrics

An accelerated laundering test method was used to evaluate the durability of the microcapsule-treated samples (AATCC Test Method 61, 2006). The microcapsules containing cotton sample was cut into 5cm × 15cm. One hundred and fifty milliliters of 0.15%w/v standard reference detergent and 50 steel balls were added into the stainless steel container together with the specimen. The containers containing the test samples were then put into the washing machine (Atlas Launder Ometer, US) operated at 49℃ for desired washing cycles. Afterwards, the washed specimens were rinsed twice for 1 minute in two separate 100mL portions of deionized water. The samples were dried at room temperature. The dried cotton fabrics were conditioned under the relative humidity of 65 ± 2% at 21 ± 1 ^{o}C for 24 hours prior to all experiments. The weight loss of microcapsules after different washing cycles was recorded as compared to the unwashed sample.

7.2.5 Surface Morphology of Microcapsule-Treated Cotton Fabrics

Surface appearance of the microcapsule-treated fabrics was evaluated as described in Chapter 3, Section 3.2.5. The SEM was operated at a high magnification up to 250X and 500X to observe the surface morphological appearance of cotton fibres after microcapsule treatments.

7.2.6 FTIR Analysis of Microcapsule-Treated Cotton Fabrics

FTIR spectrum of microcapsule-treated cotton fabrics was analyzed as described in Chapter 3, Section 3.2.6. The significant peak of berberine loaded microcapsules representing the aromatic C=C vibrations at 1505cm^{-1} was investigated. The peak areas were also calculated in order to provide a quantitative analysis of those microcapsules on cotton fabrics after various washing cycles (Monllor *et al.*, 2007).

7.2.7 Antibacterial Assessment of Cotton Fabrics Containing Berberine Loaded Microcapsules

The antibacterial study of microcapsule-treated cotton fabrics was performed as described in Chapter 6, Section 6.2.10.

7.3 Results and Discussion

7.3.1 Surface Morphology

Figure 7.1 and 7.2 show the SEM images of BER/CS microcapsule and BER/AG microcapsule containing cotton samples after 5, 10, 20, 40 and 50 washing cycles. After 5 and 10 washing cycles as shown in Figure 7.1B and 7.1C, and Figure 7.2B and 7.2C, a majority of microcapsules still tightly adhered on cotton fibre surface. After 20 washing cycles (Figure 7.1D and 7.2D), a certain amount of

microcapsules still appeared on the fibre surface but with some breakages due to certain agitation during washing and laundering. It was noticed that the binding ability between microcapsules and cotton fibres was considered to be satisfactory after 20 washing cycles. However, after 40 and 50 washing cycles as shown in Figure 7.1E and 7.1F, and Figure 7.2E and 7.2F, little amount of microcapsules was found on the fibre surface because of significant decrease in the binding ability between microcapsules and cotton fibres during repeated washing and laundering processes.

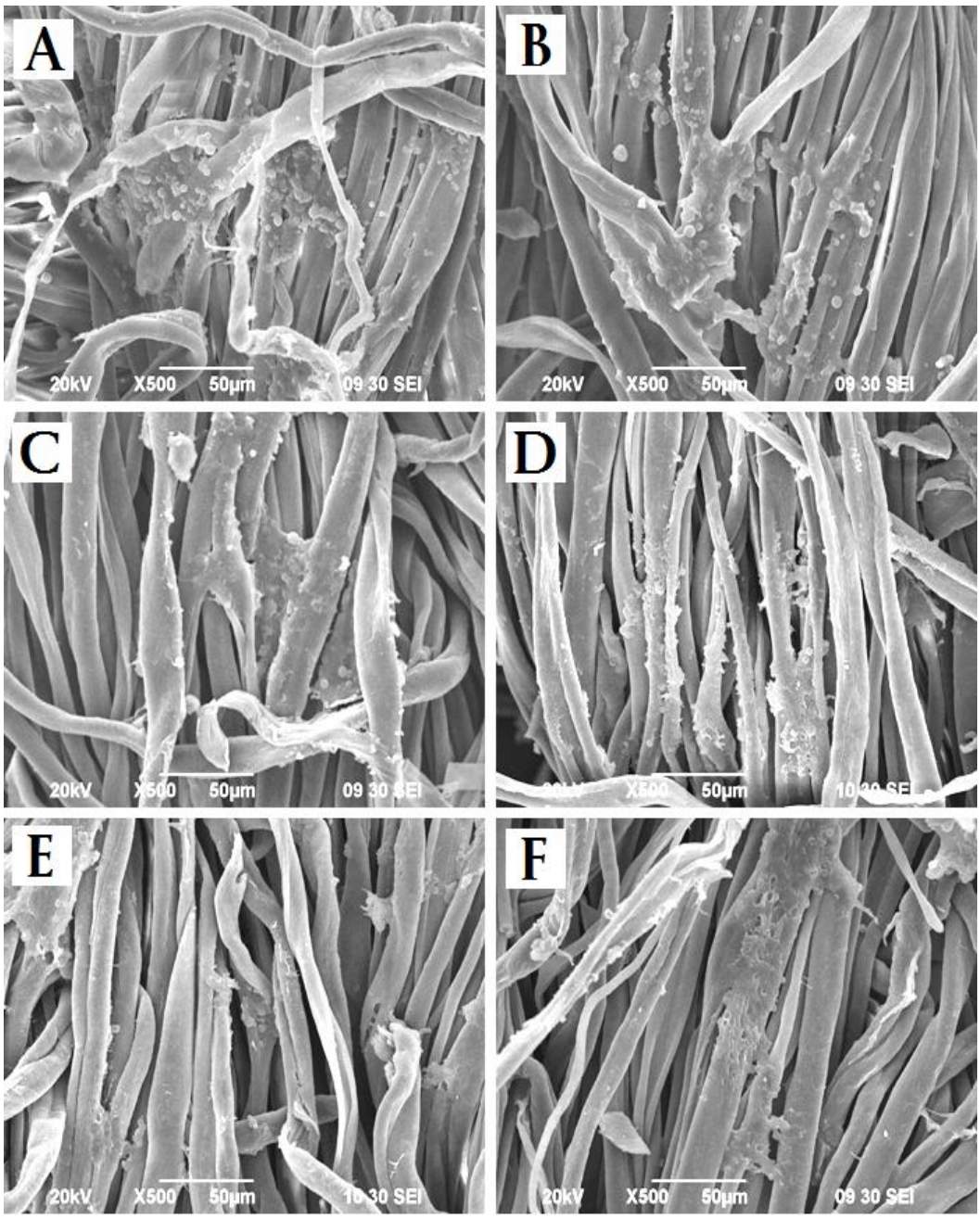


Figure 7.1 SEM images of BER/CS microcapsule containing cotton fabrics after various washing cycles: (A) unwashed, (B) 5 cycles, (C) 10 cycles, (D) 20 cycles, (E) 40 cycles and (F) 50 cycles

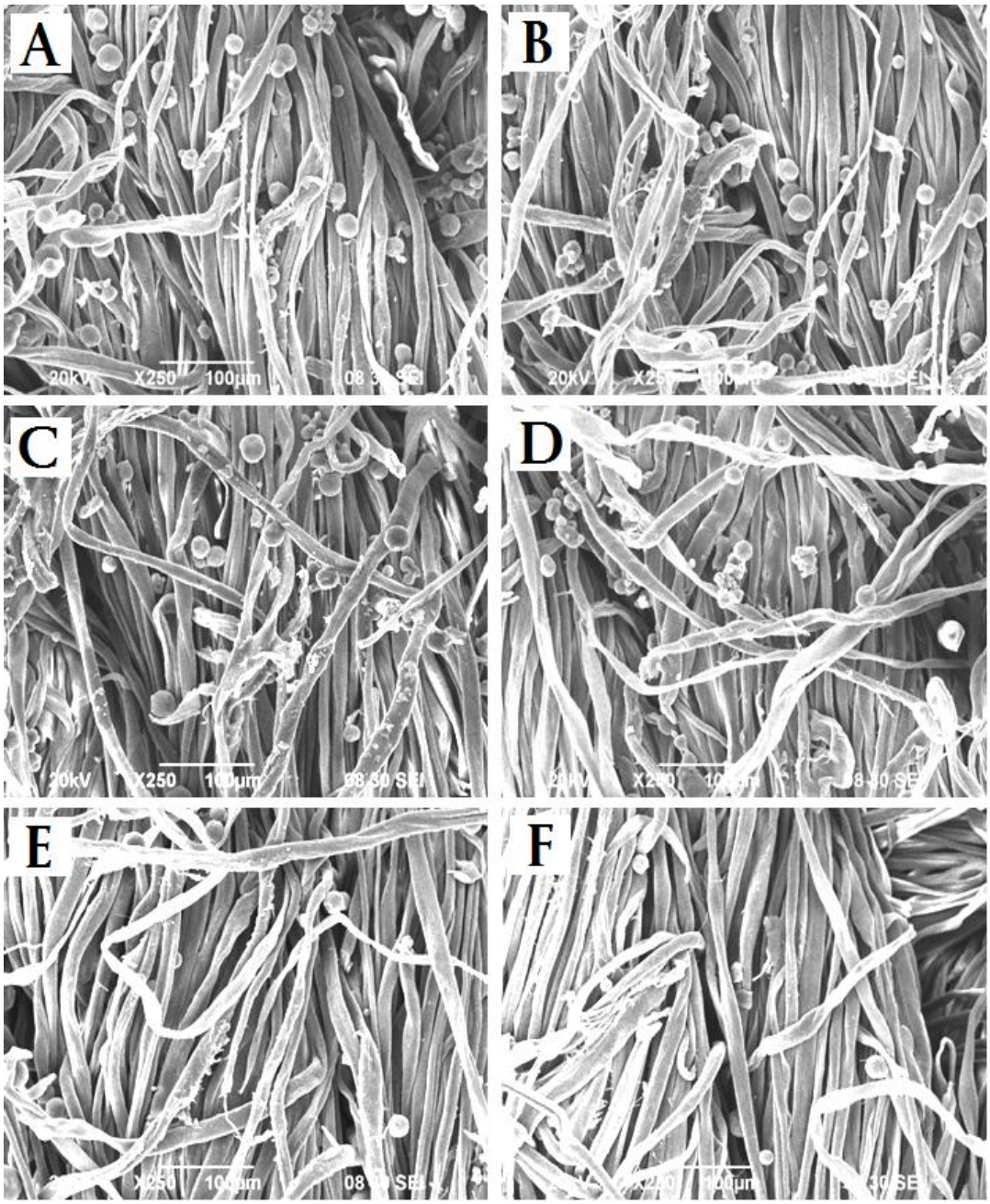


Figure 7.2 SEM images of BER/AG microcapsule containing cotton fabrics after various washing cycles: (A) unwashed, (B) 5 cycles, (C) 10 cycles, (D) 20 cycles, (E) 40 cycles and (F) 50 cycles

7.3.2 Washing Fastness of Microcapsule-Treated Cotton Fabrics

Figure 7.3 demonstrates the weight loss of microcapsules after various washing cycles. It was observed that the weight loss of microcapsules was higher than the cotton fabrics treated with BER/CS microcapsules and that of BER/AG microcapsules treated samples. The washing test was consistent with the SEM results. An increment in washing cycles led to higher loss of microcapsules during the washing and laundering processes.

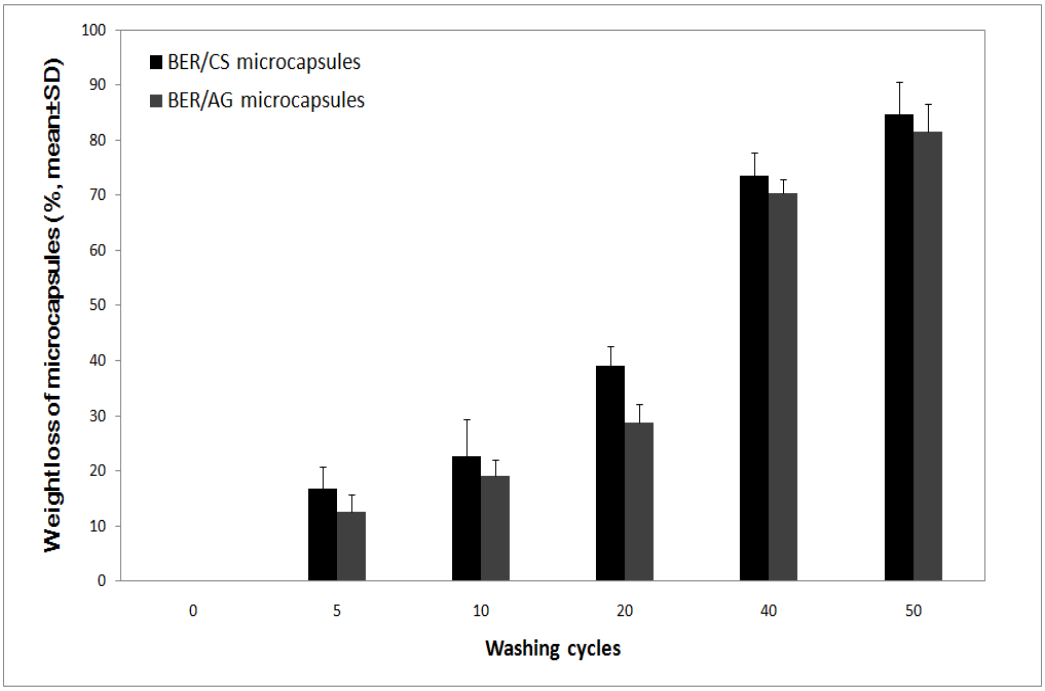


Figure 7.3 Weight loss of microcapsules after various washing cycles

7.3.3 FTIR Analysis

The amount of berberine containing microcapsules attached on cotton fabrics was decreased with the increment in washing cycles. In this paper, changes in the aromatic C=C region at 1505cm^{-1} which existed in berberine was investigated for microcapsule-treated cotton samples with various washing cycles. From Figure 7.4 and 7.5, it was noticed that control cotton samples did not contain the

aromatic C=C region while all of the microcapsule-treated cotton fabrics had the significant peak at 1505cm^{-1} which indicated the existence of aromatic C=C region from berberine. It was observed that the amount of berberine containing microcapsules of both cotton samples decreased with the increment in washing cycles. The amount of berberine containing microcapsules in BER/AG microcapsule-treated cotton fabrics was more than that in BER/CS microcapsule-treated samples even after several washing cycles. The result was consistent with that of weight loss in microcapsule-treated fabrics.

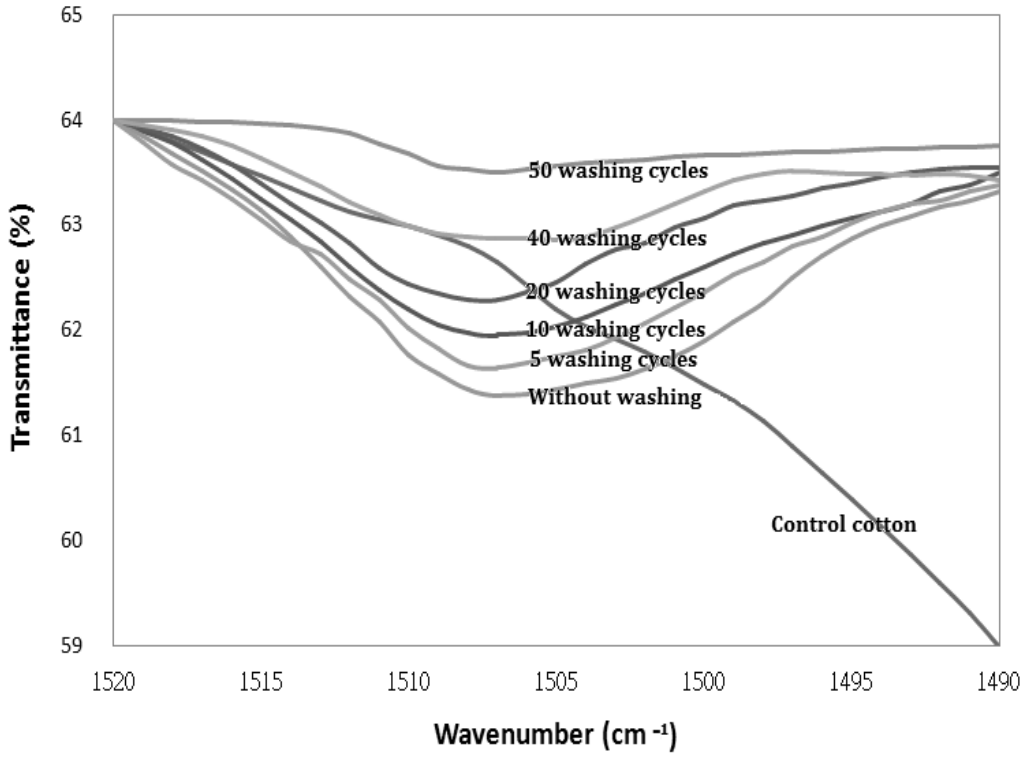


Figure 7.4 FTIR spectrum of BER/CS microcapsule-treated cotton fabric after different washing cycles

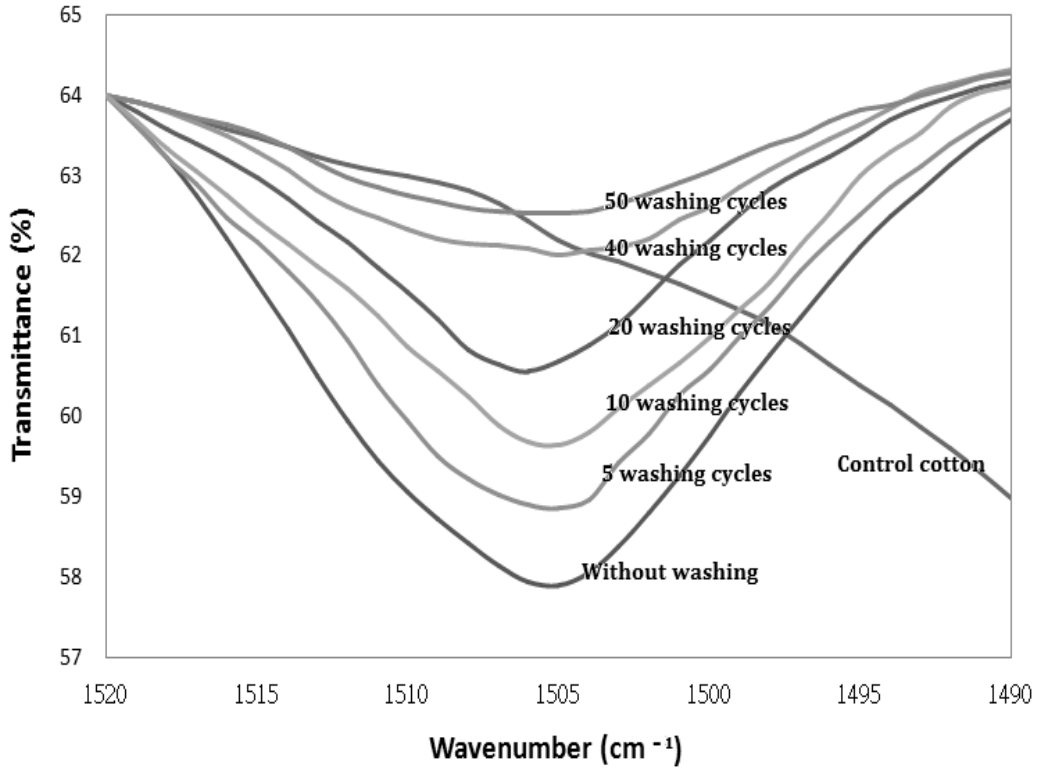
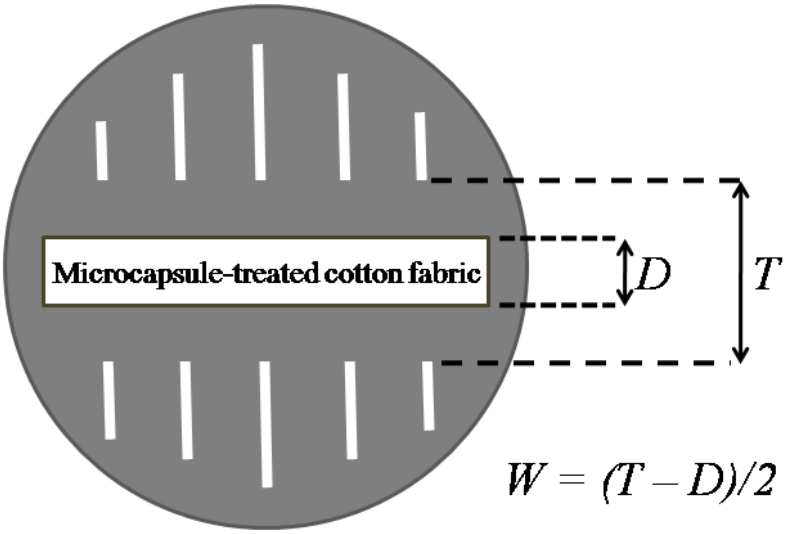


Figure 7.5 FTIR spectrum of BER/AG microcapsule-treated cotton fabric after different washing cycles

7.3.4 Antibacterial Study

Figure 7.6 demonstrates the Schematic diagram of microcapsule-treated cotton fabric with clear zone of inhibition towards *S. aureus*. From Figure 7.7, it was observed that BER/AG microcapsule-treated cotton samples (Figure 7.7A) exhibited a stronger growth inhibition towards *S. aureus* even after 20 washing cycles when compared with that of BER/CS microcapsule-treated fabrics (Figure 7.7B). Table 7.1 indicates that the zone of clearance of BER/AG microcapsule-treated specimens decreased with the increment in washing cycles. The anti-*S. aureus* activity of that samples can be lasted for 20 washing cycles. On the other hand, it was found that BER/CS microcapsule-treated cotton fabrics only showed the bacterial inhibition in the contact area until 20 washing cycles.

This might be due to the fact that berberine could be easily released via an internal water phase of BER/AG microcapsules. Conversely, it was comparatively difficult for berberine to be released from an interior oil phase of BER/CS microcapsules. With more and more amount of microcapsules losing in the repeated washing and laundering processes, the antibacterial activity of both microcapsule-treated fabrics gradually diminished.



where:
W = width of clear zone of inhibition in mm
T = total diameter of test specimen and clear zone in mm
D = diameter of the test specimen in mm

Figure 7.6 Schematic diagram of microcapsule-treated cotton fabric with clear zone of inhibition towards *S. aureus*. Clear in contact area refers to the area between the microcapsule-treated cotton fabric and the corresponding agar surface with no growth of *S. aureus*

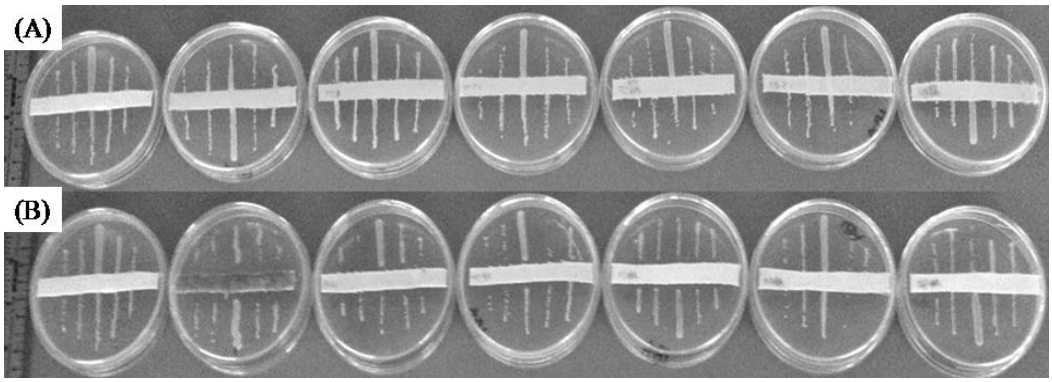


Figure 7.7 Growth inhibition of microcapsule-treated cotton fabrics towards *S. aureus*: (A) BER/CS microcapsule-treated samples, (B) BER/AG microcapsule-treated samples. For (A) and (B), the samples from left to right represent control cotton, 0, 5, 10, 20, 40 and 50 washing cycles

Table 7.1 Zone of clearance (mm) of BER/CS microcapsule-treated samples and BER/AG microcapsule-treated samples towards *S. aureus*

	Washing cycles					
	0	5	10	20	40	50
(A). BER/CS microcapsules treated sample	clear in contact area	clear in contact area	clear in contact area	clear in contact area	Nil	Nil
(B). BER/AG microcapsules treated sample	4.33 ± 0.29	3.33 ± 0.58	2.50 ± 0.50	2.17 ± 0.29	Nil	Nil

7.4 Conclusion

The performance of core active compounds could be affected by the internal phase of microcapsules. In this study, two microencapsulation systems including a chitosan-based (oil-in-water) system and an agar-gelatin based (water-in-oil) system containing berberine were fabricated onto cotton fabrics so as to promote an anti-*Staphylococcus aureus* (*S. aureus*) activity for textile

materials. The mean drug loading efficiency of BER/CS microcapsules and BER/AG microcapsules was estimated to be 51.36% ± 2.31 and 78.16% ± 2.60 respectively whereas the average particle size of these microcapsules was 5.27μm ± 1.65 and 16.75μm ± 4.54 respectively. The SEM images, weight loss study, FTIR analysis and anti-*S. aureus* assay demonstrated that the agar-gelatin microcapsules containing the internal hydrohilic phase of berberine maintained relatively higher amount on cotton fabrics and exhibited better antibacterial activity even after 20 washing cycles when compared with that of chitosan microcapsules having the core oil phase of berberine. The results revealed that the core aqueous phase of berberine was more easily to be released from the microcapsules and processed its anti-*S. aureus* activity.

CHAPTER 8

CONCLUSION AND FUTURE RECOMMENDATION

8.1 Conclusion

In this thesis, an oil-in-water (O/W) microencapsulation system was developed using chitosan as a wall shell material while a free-formaldehyde water-in-oil (W/O) microencapsulation system was developed using agar and gelatin as the wall matrix materials. Different drugs or biomedical agents were encapsulated into the wall shell or wall matrix of microcapsules in order to promote the prolonged therapeutic effects and pharmaceutical functions. The use of the developed chitosan-based and agar/gelatin-based microencapsulation systems containing biomedical agents is believed to offer an alternative route for both oral and topical drug deliveries. The present research attempts to give a comprehensive development and systematic characterization of microencapsulated drug development for both oral and transdermal applications.

8.1.1 Development of Chitosan-Based System for Both Oral and Topical Drug Deliveries

With reference to the O/W chitosan-based system, chitosan-based microcapsules were prepared with chitosan as a wall shell material and calendula oil as the core substance using the simple coacervation technique. By analyzing the encapsulation efficiency and mean particle size, the optimal parameter combinations were achieved as follows: concentration of chitosan: 1.5%w/v; core/wall ratio: 0.1g/ml; stirring speed: 1200rpm; pH value: 10. By using these optimal conditions, the encapsulation efficiency and the mean particle size were 82.37% and 3.78 μm respectively. The physical properties of optimal calendula

oil loaded chitosan microcapsules were examined by SEM images, particle size analysis, FTIR spectrum, *in vitro* release study as well as *in vitro* and *in vivo* toxicology analysis. The *in vitro* cytotoxicity evaluation demonstrated that the microcapsules exhibited no significant cytotoxic effects on human keratinocyte skin cells at a maximum concentration of 100 mg/mL. The calendula oil containing chitosan microcapsules were therefore shown to be non-cytotoxic towards the human HaCaT cells. The *in vivo* toxicology analysis on mice further demonstrated that when the microcapsules were administrated orally at 20 mg of microcapsules per gram of body weight of mice or 2 mg of oil per gram of body weight did not show any significant damage to the liver section. Both toxicity results suggested that the calendula oil/ chitosan microcapsules could be applied both orally and topically without any adverse cytotoxic effects. The microcapsules were also fabricated onto cotton fabrics and the released calendula oil from microcapsules was believed to promote the health benefits during daily wearing clothes.

After confirming the safety issues of the developed calendula oil containing chitosan microcapsules, it was further demonstrated that the possibility of the oral application was investigated using hydrocortisone succinic acid (HSA) microcapsules while the possibility of topical usage was examined using 5-fluoruracil (5-FU) and phyllanthin microcapsules. The use of polymeric microcapsules to encapsulate the drugs for both oral and topical applications was believed to be helpful in drug deliveries. Microencapsulated drugs were proposed to be gradually released under control from microcapsules and the sustained release could promote a longer lasting effect of core active drugs. In the present study, all drugs were confirmed to be entrapped into the chitosan-based

microcapsules using both UV VIS spectrophotometer and FTIR analysis. *In vitro* release study revealed that all drugs could be gradually released from the microcapsules over time. The mean ACTH concentration in HSA loaded microcapsule mice plasma was detected to be about 42.2% lower than that of water control. It was also revealed that microcapsules containing 100μg/mL of 5-FU led to a better cell growth inhibition of human keratinocytes when compared to that of 5-FU. *In vitro* drug delivery model also demonstrated that 5-FU could be released from microcapsule treated textile materials on the intact skin model. *In vitro* biological assays revealed that these phyllanthin containing microcapsules showed a stronger anti-oxidation potential on both human fibroblasts and keratinocytes as well as a better growth inhibitory activity towards *S. aureus*. As a result, it is speculated that the developed chitosan-based microcapsules could be a versatile drug delivery vehicle for both oral and topical medications.

8.1.2 Development of Agar/Gelatin-Based System for Both Oral and Topical Drug Deliveries

Apart from the chitosan (O/W) microencapsulation system for both oral and topical drug deliveries, a free-formaldehyde water-in-oil (W/O) system was also developed using agar and gelatin as the wall matrix materials of microcapsules. This study reported the development, characterization and safety uses of agar/gelatin microcapsules. Generally, formaldehyde is commonly applied as a crosslinker for gelatin microcapsulation in many previous studies. Formaldehyde is important to strengthen the wall matrix of gelatin-based microcapsules through the crosslinking process. However, it is considerably toxic to the human body. Therefore, the present study aimed at developing a formaldehyde free approach

by employing agar together with gelatin as the wall matrix materials of microcapsules in order to offer an alternative way for microcapsule formation. It was proposed that the drug was distributed into the agar/gelatin wall matrix of microcapsules for sustained release. The developed microencapsulation recipe without introducing formaldehyde could produce the desired properties of microcapsules due to its safety and cost-efficient benefits in microcapsule formation.

The oral usage was examined using gallic acid loaded microcapsules while the topical application was tested using berberine loaded microcapsules. Microcapsules containing both drugs were prepared combining the optimal parameters identified. All drugs were confirmed to be entrapped into the chitosan-based microcapsules using both UV VIS spectrophotometer and FTIR analysis. *In vitro* release study revealed that all drugs could be gradually released from the microcapsules. The *in vivo* mice disease model also revealed that gallic acid loaded microcapsules were helpful in the treatment of acute liver and kidney toxicity. The MICs and antibacterial tests also proved that berberine loaded microcapsules exhibited a better growth inhibition activity towards *S. aureus* when compared with those of original drug. *In vitro* drug delivery model also demonstrated the delivery of berberine from microcapsule treated textiles on nude mice skin. Therefore, the development of agar/gelatin-based microcapsules was demonstrated to be an efficiency and safe deliverable tool for both oral and topical applications.

8.1.3 Washing Durability of Berberine Containing Microcapsule-Treated Cotton Fabrics

The performance of core active compounds could be affected by the internal phase of microcapsules. In this study, two microencapsulation systems including a chitosan-based (oil-in-water) system and an agar-gelatin based (water-in-oil) system containing berberine were fabricated onto cotton fabrics so as to promote an anti-*Staphylococcus aureus* (*S. aureus*) activity for textile materials. The SEM images, weight loss study, FTIR analysis and anti-*S. aureus* assay demonstrated that the agar-gelatin microcapsules containing the internal hydrohilic phase of berberine maintained relatively higher amount on cotton fabrics and exhibited better antibacterial activity even after 20 washing cycles when compared with that of chitosan microcapsules having the core oil phase of berberine. The results revealed that the core aqueous phase of berberine was more easily to be released from the microcapsules and processed its anti-*S. aureus* activity.

8.1.4 Comparison between Chitosan-Based System and Agar/Gelatin-Based System

By comparing the chitosan-based (O/W) system with the agar/gelatin-based (W/O) system, it is believed that the chitosan-based microencapsulation system might be more suitable for oral drug delivery while the agar/gelatin-based system could be relatively acceptable for topical medications. The agar/gelatin-based W/O system allowed the sustained release of internal water-soluble drugs by attaching on the skin and permitting diffusion of the therapeutic drugs, through the skin layer, into the peripheral circulation. The *in vitro* release study demonstrated that the drugs could be released at a higher rate in the

agar/gelatin-based system (around 70% of drug release for berberine containing microcapsules) when compared with chitosan-based system (around 60% of drug release for 5-FU containing microcapsules). The washing fastness study also revealed that agar/gelatin-based microcapsules containing the internal water phase of berberine maintained relatively higher amount on cotton fabrics and exhibited better antibacterial activity even after 20 washing cycles when compared with that of chitosan microcapsules having the core oil phase of berberine.

However, chitosan has an excellent mucoadhesion and it is positively charged that might increase residual time at the site of adsorption and also prolong the contact time between positively charged chitosan and negatively charged mucin in cell membrane, resulting in the improvement of drug bioavailability (Tiyaboonchai, 2003). Chitosan mucoadhesion might significantly increase the half time of its clearance (Soane *et al.*, 1999). The formed chitosan wall shell of microcapsules might have higher mucoadhesive ability than that of agar and gelatin. Agar is an anionic polymer while gelatin is an amphoteric polymer that simultaneously having cationic and anionic functional groups. Gelatin was reported to have relatively poor mucoadhesive characteristics like non-ionic polymers due to its amphoteric property and self-neutralization of cationic and anionic charged within its structure (Khutoryanskiy, 2011). Therefore, chitosan is considered as a safe material for microcapsule-based oral drug delivery carriers that protect the liable drug from the gastrointestinal environment.

8.2 Recommendations for Future Work

Oral administrated microcapsules containing drugs could be further studied using the disease model. Daunorubicin could be used as a model drug and then entrapped into the chitosan-based (O/W) microencapsulation system for oral drug delivery. Daunorubicin is a commonly used anti-cancer drug for the treatment of breast and lung carcinoma, leukemia and lymphoma. It can slow and stop the growth of cancer cells in the human body. However, daunorubicin should be administered in a rapid intravenous infusion. It should not be administered intramuscularly because it may lead to extensive tissue necrosis and must not be administered intrathecally since this may result in extensive damage to the nervous system and may cause death (Mortensen *et al.*, 1992; McBride *et al.*, 2001). Oral administration is also unavailable for daunorubicin as it may damage the esophagus, stomach as well as other organs when it is being taken orally. Therefore, it is believed that the internal oil phase of microcapsules might protect the daunorubicin from the gastrointestinal environment during oral administration. *In vivo* studies using the nude mice could be performed so as to evaluate the possibility of oral administration of the microencapsulated daunorubicin. The lymphoma cancer cells will be injected to the nude mice body for tumor development. Afterwards, the effect of drug loaded microcapsules on the lymphoma treatment could be further investigated in future.

Antibiotics could be applied to the agar/gelatin (W/O) microencapsulation system for transdermal drug delivery. In fact, numerous people have suffered from the skin bacterial infection in the world. Antibiotic is an antagonistic agent that inhibits the growth of microorganisms. However, frequent use of antibiotics can lead to the emergency of bacterial resistance to the antibacterial drugs.

During the antibacterial treatment, susceptible bacteria are inhibited by the drug but some stronger bacteria may still survive and result in an inheritable resistance. Therefore, some antibiotics could be encapsulated into the wall matrix of microcapsules in order to modify the drug resistance. The influence of sustained drug release on the growth inhibition towards microorganisms could be investigated. *In vitro* assessment on the microorganisms obtained from the skin infection suffered patients and *in vivo* clinical trials on human beings could be examined in future.

Biomedical textiles is now rapidly developed in textile industry. Textiles with therapeutic benefits can be promoted by microencapsulation technology. The use of polymeric microcapsules to encapsulate the biomedical agents and commercial drugs is capable of achieving the therapeutic and medical purposes to heal some human ailments and diseases. The medicines or biomedical ingredients are released under control from the microcapsules in order to prolong the life cycle of drugs and maximize the therapeutic actions to human beings. Antimicrobial textiles is one of the rapidly growing areas in the world textile industry. This type of textiles can be applied to a variety of medical and hygienic products used in hospitals in order to provide an antimicrobial protection for the patient environment. Since hospital environments can be contaminated with pathogens including bacteria and fungi easily, antimicrobial agent containing microcapsules could be further developed and then fabricated onto the textile materials in order to develop the antimicrobial textiles for hospital environments. The antimicrobial textiles could be used in surgical garments, bedclothes and hospital uniforms so as to provide a highly hygienic condition and a stronger protection from microorganisms in hospital environments. The active

antimicrobials of the medical textiles are released under friction and pressure during human wear, people can then experience the biomedical and healthcare benefits via a simple daily wear.

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