ANTI-CANDIDAL ACTIVITY AND TOXICITY STUDIES OF ALGINATE-ENCLOSED CHITOSAN–CALCIUM PHOSPHATE-LOADED FE-BOVINE LACTOFERRIN NANOCAPSULES

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LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bLf	Bovine lactoferrin
CC_{50}	50% Cytotoxic concentration
CFU	Colony forming unit
CLSI	Clinical & Laboratory Standards Institute
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	50% Effective Concentration
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
H&E	Haematoxylin and Eosin
H_2O_2	Hydrogen peroxide
IC_{50}	50% Inhibitory concentration
i.p.	Intraperitoneal
i.v.	Intravenous

kDa	Kilodalton
LC_{50}	Lethal concentration
MIC	Minimal inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NCs	Alginate/EUDRAGIT® S 100-enclosed chitosan–calcium phosphate-loaded Fe-bLf nanocapsules
OD	Optical density
PBS	Phosphate buffer saline
SDA	Sabouraud dextrose agar
SEM	Scanning electron microscope
TEM	Transmission electron microscope

LIST OF APPENDICES

AKTIVITI ANTIKANDIDA DAN KAJIAN TOKSISITI NANOKAPSUL BERISI FE-BOVIN LAKTOFERIN KITOSAN-KALSIUM FOSFAT DISELAPUTI ALGINAT

ABSTRAK

Laktoferin diketahui mempunyai pelbagai aktiviti antimikrob. Penyelidikan ini dijalankan untuk mengkaji aktiviti antikandida dan toksisiti secara *in vitro, in vivo* nanokapsul Fe-Lfb berisi kitosan-kalsium fosfat diselaputi Alginat/EUDRAGIT®S 100 (NCs). Penyaringan aktiviti antimikrob NCs menunjukkan bahawa NCs mempunyai aktiviti antikandida yang baik. Kepekatan perencatan minimum (MIC) NCs yang diperolehi adalah 500 µg/mL. Kajian profil pertumbuhan menunjukkan bahawa NCs boleh merencatkan pertumbuhan *Candida albicans* USM-K1 (*C. albicans*) pada kepekatan MIC, dua kali MIC dan empat kali MIC. Kajian elektron mikroskopi menunjukkan NCs menyebabkan kemusnahan sel-sel yis secara gangguan terhadap dinding sel dan membran sel dengan peningkatan masa pengeraman sehingga 36 jam. NCs juga merencatkan pertumbuhan tiub-tiub germa *C. albicans* USM-K1. Kajian kokultur sel-sel epitelium dengan *C. albicans* menunjukkan bahawa NCs meningkatkan pembunuhan *C. albicans* oleh sel-sel epitelial mulut. Kajian *in vivo* menunjukkan NCs berkeupayaan menyebabkan pengurangan unit pembentukan koloni (CFU) sampel ginjal mencit yang dijangkiti yis daripada $(21.3 \pm 1.3) \times 10^4$ ke $(4120.0 \pm 6.4) \times 10$ ($p < 0.05$). Ini menunjukkan bahawa NCs mempunyai kesan antikandida terhadap *C. albicans* secara *in vivo*. Pemeriksaan histologi menunjukkan sampel ginjal mencit yang dirawat dengan NCs mempunyai struktur dan koordinasi sel yang lebih baik berbanding dengan mencit

yang tidak menerima rawatan. Kajian toksisiti dengan menggunakan anak udang brin menunjukkan nilai LC_{50} bagi NCs adalah melebihi 1 mg/mL yang menunjukkan bahawa ia adalah tidak toksik. Kajian sitotoksisiti dengan menggunakan kaedah MTT juga menunjukkan NCs adalah tidak toksik terhadap sel-sel Vero bukan kanser. Kajian genotoksiti melalui ujian komet terhadap sel-sel Vero dan ujian akar *Allium cepa* menunjukkan bahawa NCs tidak mempunyai kesan genotoksik yang signifikan pada keadaan kajian yang ditetapkan. Sebagai kesimpulan, NCs adalah agen antikandida yang berkesan dan berpotensi sebagai agen terapi untuk kandidiasis.

ANTI-CANDIDAL ACTIVITY AND TOXICITY STUDIES OF ALGINATE-ENCLOSED CHITOSAN–CALCIUM PHOSPHATE-LOADED FE-BOVINE LACTOFERRIN NANOCAPSULES

ABSTRACT

Lactoferrin has been known to have antimicrobial properties. This research was conducted to investigate the anti-candidal activity and toxicity of Alginate/EUDRAGIT® S 100-enclosed chitosan–calcium phosphate-loaded Fe-bLf nanocapsules (NCs) by *in vitro* and *in vivo* study. The screening of NCs for antimicrobial activity showed that NCs has good anti-candidal activity. The minimum inhibitory concentration (MIC) value of NCs using broth microdilution method was found to be 500 µg/mL. The growth profile study demonstrated that the NCs at MIC, two times MIC and four times MIC inhibited the growth of *Candida albicans* USM-K1 (*C. albicans*). The electron microscopy studies showed that NCs caused the destruction of yeast cells by disruption of cell wall and cell membrane with increase of incubation time until 36 hours. NCs also inhibited germ tube formation of *C. albicans*. Evaluation of vital staining of epithelial cell and *C. albicans* coculture demonstrated that NCs enhanced the killing of *C. albicans* by epithelial cells. *In vivo* studies showed that NCs caused the reduction of colony forming unit (CFU) of kidney samples of mice induced with yeast infection from $(21.3 \pm 1.3) \times 10^4$ to $(4120.0 \pm 6.4) \times 10$ ($p < 0.05$). This shows that NCs have *in vivo* anticandidal effect. Histological examination of kidney samples of mice treated with NCs showed better cell structure and coordination compared to untreated mice. Brine shrimp lethality assay showed that the LC_{50} value of NCs was more than 1 mg/mL

which indicated that NCs was not toxic. MTT cytotoxicity assay also revealed that NCs was not toxic against non-cancerous Vero cell line. Genotoxicity studies by comet assay on Vero cells and *Allium cepa* root assay revealed that the NCs was destitute of significant genotoxic effect under experimental conditions. In conclusion, NCs has effective anticandidal properties and has the potential as a therapeutic agent against candidiasis.

CHAPTER 1

INTRODUCTION

1.1 Overview and Rational of Study

Pathogenic fungi such as *Candida albicans* are a key contributory agent for opportunistic infections. Immunocompromised individuals such as AIDS patients are at danger for opportunistic infections, which if not cured properly, contribute to the death linked to their disorders. Inadequate availability of effective antifungal drugs and the increasing prevalence of this life threatening fungal infection (Magliani *et al*., 2012) have conspired to outgrowth a necessity for developing novel treatment options for opportunistic infections by pathogenic fungi such as *C. albicans*. Fungal cells which categorised as eukaryotes shared numerous similarities with mammalian cells such as the nucleus contains DNA organized into chromosomes with distinct cytoplasmic organelles and biosynthetic pathways similar to mammalian cells. These likenesses have made extra problems in the design of antifungal agent with selective toxicity to fungal cells (Enoch *et al*., 2006; Martins *et al*., 2011). Moreover, fungal cells, like various microorganisms are surrounded by cell wall consist of β-glucan, chitin and mannoproteins which offer physical support and defence to the cell develop additional obstacle in the design of antifungal agent (Georgopapadakou & Tkacz, 1995). The presence of fungal cell wall which is a complex of biopolymers made the discovery of antifungal agents remains an important scientific challenge. The insoluble polysaccharides in cell wall convey mechanical strength to the fungal cell (Cabib *et al*., 1988). Hence, the fungal cell wall is emerging as an important better defined therapeutic target. For example, in *C. albicans*, it has been proposed

that β-glucan and chitin are related to the strength and shape of the cell wall, while mannoproteins are accountable for the porosity, its antigenicity and adhesion (Cabib *et al*., 1998). As a result, no antifungal drug was available for the cure of fungal infections until the encounter of amphotericin B in 1953 (Dutcher, 1968). This was followed by the development of many new antifungal agents. However, with the advent of the immunocompromised diseases such as AIDS, the growing number of patients alive with repressed immune systems and the deficiency of effective antifungal agent for the cure of fungal infections, there has been renaissance in the awareness in the search for new, safer, and more efficacious antifungal agents to battle serious fungal infections (Barrera *et al*., 2014; Knechtle *et al*., 2014).

Concomitant with this, most of the consideration has been dedicated to the development of an ideal antifungal agent. Therefore, an ideal antifungal agent should possess a few characteristics such as target specific, has multiple mode of delivery, mainly oral delivery with minimal toxicities or side effects. It is clear that designing an antifungal drug that fulfils the above criteria is a key challenge and requires the identification of new biological natural products such as lactoferrin. Lactoferrin is a multifunctional iron-binding glycoprotein belonging to the transferrin family present in mammalian milk. It has also been shown to inhibit a wide range of fungal, bacterial, viral, and parasitic pathogens (Jenssen & Hancock, 2009; Leboffe *et al*., 2009; Berlutti *et al*., 2011; Giansanti *et al*., 2013). The anticandidal mode of action of lactoferrin was suggested to be due to cell wall perturbation (Jenssen & Hancock, 2009), as proved by cryo-scanning electron microscopy study which showed the drastic changes in the cell wall, leading to the formation of surface blebs, swelling and cell collapse (Xu *et al*., 1999). Similar damages on cell wall were also reported

by Nikawa *et al*. (1993; 1995) after *Candida* exposure to both human and bovine lactoferrin and it was concluded that the anticandidal activity of lactoferrin is due to direct contact of the protein with the fungal cell surface (Valenti *et al*., 1985).

Between the numerous ways of drug administration, oral drug delivery is marked as the harmless. But, for development of novel drug molecule there are a lot of problems that has to be tackled when drugs are being delivered through gastrointestinal tract of human. These include enzymatic action and gut pH, which may lead to degradation of this protein. Recent advances in nanotechnology has facilitate oral delivery system for better efficacy, such as slow drug delivery, target specificity, better efficacy, and less side effects, which are crucial for drug design and development. For the past few decades, various formulations of nanoparticles have been used to increase therapeutic benefit (Samarasinghe *et al*., 2012). Earlier successful studies have shown efficacy of the bovine lactoferrin against various microorganism (Rogan *et al*., 2006; Mahidhara *et al*., 2015). Numerous studies that have recognised the role of lactoferrin molecules against bacteria, viruses, and fungal infections (Baker & Baker, 2005).

Recently, Kanwar *et al*. (2015) has prepared alginate chitosan calcium phosphate nanocapsules (AEC-CCo-CP-NCs), which have been widely used as drug carriers. Alginate and chitosan are biodegradable polymers, and calcium phosphate is naturally present in teeth therefore are safe to be used as NCs.

In current study, the *in vitro* and *in vivo* experiments were carried out using an encapsulated Fe-bovine lactoferrin (AEC-CP-Fe-bLf NCs) preparation to

investigate its potential in the treatment of *C. albicans*. The Alginate-enclosed chitosan-calcium phosphate-loaded Fe-bovine lactoferrin nanocapsules (NCs) were prepared by a combination of nano precipitation and ionic gelation methods by Kanwar *et al*. (2012) with modifications. These NCs have been loaded with bovine lactoferrin molecule to protect it from degradation of gut environment. Therefore, bioavailability of lactoferrin protein at the site of microbial infection was not altered and will show maximum antimicrobial efficacy. Content of Fe-bLf were analysed from tissue lysates which showed maximum Fe-bLf concentrations in tumor (two to four-fold higher), bone (twofold higher), blood (twofold higher) and intestines (twofold higher). These findings demonstrated maximum distribution of fe-bLf and NCs in muscle, blood, liver, bone, spleen, kidney, lung and intestine (Kanwar *et al*., 2015). The antimicrobial efficacy of NCs has been studied *in vivo* against pathogenic *Salmonella* Typhimurium using Balb/c mice model (Gupta *et al*., 2015). These success stories have originated the idea to use NCs in treatment of *C. albicans* infections.

Candidiasis is a fungal infection most commonly caused by the dimorphic fungus *C. albicans*. In contrast to other fungal pathogens, *C. albicans* is a member of the normal flora in the gastrointestinal tract (mouth and oropharynx), respiratory system and vaginal area. The growth of *C. albicans* is suppressed by other normal flora in the body. However, if the normal flora is being disturbed, *Candida* can multiply rapidly and cause candidiasis. In recent years, *Candida* species have also been identified as nosocomial pathogens (Prescott *et al*., 2003). Besides that, *Candida* can also be transmitted sexually. A majority of candidal infections involves

the skin or mucous membranes. This is because *C. albicans* is a strict aerobe and therefore such surfaces are suitable for its growth.

Previously, lactoferrin was reported to possess antifungal properties against *C. albicans* by Kirkpatrick *et al*., (1971). Oral candidiasis is linked with impaired secretion of lactoferrin by the salivary glands. Patients had as much as 65% lactoferrin reduction compared to healthy individuals. Nanoformulation of lactoferrin offers various advantages such as sustained release, better efficiency, lesser side effects, target specificity and improved delivery (Kanwar *et al*., 2011). NCs loaded with lactoferrin have never been tested against *C. albicans* by previous researchers;. The present study is therefore represent the first study to investigate the effect of NCs against *C. albicans*. Specifically, this work was to analyse the effect of nanoformulated (AEC-CCo-CP-bLf-NC) bovine lactoferrin against *C. albicans*.

Various formulations of nanoparticles are being used as a novel delivery system for drugs, proteins, DNA and monoclonal antibodies (Nowrouzi *et al*., 2010; De Jong *et al*., 2008; Lewinski *et al*., 2008). Nevertheless, challenges must be overcome if the application of nanoformulation is to increase therapeutic benefit and to yield improved therapies. In view of the potential applications of nanoformulation in the pharmaceutical sector and the rising worries of United States Food and Drug Administration (FDA) about the toxic potential of nanoproducts, there is a need to study the toxicological effects of nanoproducts. In this research, the toxicity of NCs was studied using brine shrimp lethality assay, *Allium cepa* assay, cell cytotoxicity assay and comet assay.

1.2 Objectives

The current study was undertaken with the following specific objectives:

(i) To evaluate the *in vitro* anticandidal activity of Alginate/EUDRAGIT® S 100-enclosed chitosan–calcium phosphate-loaded Fe-bLf nanocapsules (NCs) against *Candida albicans*;

(ii) To study the effect of NCs on the morphology of *C. albicans* by using scanning and transmission electron microscopy;

(iii) To evaluate *in vivo* and *in vitro* cell cytotoxicity of the NCs by using brine shrimp lethality assay and MTT assay;

(iv) To evaluate the *in vitro* and *in vivo* genotoxicity of the NCs by comet assay and *Allium cepa* assay, and

(v) To evaluate the *in vivo* antiactivity of *C. albicans* by NCs in an animal model.

CHAPTER 2

LITERATURE REVIEW

2.1 Candidiasis

Candidiasis is the most common fungal infection caused by *Candida* species (Hoepelman & Dupont, 1996). Candidiasis include superficial infections of the skin, nails and mucous membranes, candidaemia and systemic infections. Vaginal candidiasis most commonly infect non-immunocompromised patients whereas superficial candidiasi,s infects mostly the oral cavity, is the most common type of infection among immunocompromised patients. Oesophagial candidiasis is another type of candidiasis which is usually related with serious immunological disorder. Oropharyngeal candidiasis is a major concern in patients infected with HIV.

The genus *Candida* comprises of several species which are pathogenic. *C. albicans* is the main species but other species such as *Candida kruseii* and *Candida parapsilosis* can also cause disease in humans. *Candida tropicalis* and *Candida stellatoida* have been reported to cause diseases in animals. *C. albicans* is a dimorphic fungus that has two growth phases (yeast and hyphal form) (Challacombe, 1994). Yeast is a fungus which reproduces by budding.

C. albicans is commonly found on mucosal membranes of the oral cavity, gastrointestinal system and vagina (Hay, 1999). Approximately 90% of yeast isolated

from the vagina is *C. albicans* and the remaining species are most commonly *Candida glabarata* and *Candida tropicalis*. Non-*C. albicans* species can cause vaginitis and is usually more resistant to conventional treatment. *Candida* has been reported to be the second most common cause of vaginal infection in the United States (Sobel, 2010). Approximately 75% of women experience vulvovaginal candidiasis at least once during their childbearing years. Besides that, hepatic candidiasis has become a more common disease among immunocompromised patients (Haron *et al*., 1987).

The occurrence of vaginal candidiasis among pregnant women is two times higher than non-pregnant women (Merkus, 1990). It has been reported that *Candida* strains can be cultured from the vagina in 10% to 20% of pregnant women and the occurrence is even higher during late pregnancy. *C. albicans* is the cause of candidiasis in 80% to 90% of the cases. During pregnancy, the treatment of candidiasis is vital for the mother to protect the fetus from life-threatening sepsis.

Itraconazole is not suitable for oral treatment of vaginal candidiasis in pregnant women due to the potential risk of malformations of the fetus. The use of broad spectrum antibacterial drugs for terminal ill patients in intensive care unit have led to candidemia and non-mucosal candidiasis in the non-HIV population (Hospenthal *et al*., 2004).

Acute pseudomembranous candidiasis or more commonly known as thrush is a common disease among young, elderly and weakened individuals. Acute atrophic candidiasis is also known as antibiotic sore mouth because of its common occurrence during the antibiotics treatments. Chronic atrophic candidiasis , caused by the colonization of *Candida* species especially among those who wear their dentures day and night. Whereas chronic hyperplastic candidiasis is common among the middle aged and elderly patients. This condition has a potential risk of cancerous transformation. Erythematous candidiasis is a type of candidiasis related to HIV infection (Challacombe, 1994). The most common form of oral candidiasis is thrush whereby there is an inflammation of oral mucosa with the formation of superficial white plaques and ulcers. Initially, the lesions are localised but later spread to entire mouth. The lesions can be asymptomatic or with pain, burning sensation and dryness of the mouth, loss of taste and difficulty in swallowing (Hoepelman & Dupont, 1996). In critically ill patients, invasive candidiasis has become an important nosocomial infection. It has been reported that *C. albicans* (>50%) is the main cause of invasive candidiasis in Taiwan (Ruan & Hsueh, 2009).

2.2 Antifungal Therapy

Various classes of antifungal agents are being employed for the treatment of fungal infections (Sanglard, 2003). Before the emergence of triazole antifungal drugs in the 1980s, the only agent to treat non-mucosal fungal infections was intravenous amphotericin B. There was no effective alternative therapy and treatment was limited to amphotericin B or without any treatment at all. The emergence of triazole

antifungal therapy, fluconazole and itraconazole enabled physicians to select alternative antifungal treatment for candidiasis. This began with the emergence of acquired immune deficiency syndrome (AIDS) in which the human immunodeficiency virus (HIV) was associated with oropharyngeal candidiasis (or thrush) and also the severity of thrush and candidal esophagitis that eventually progressed to AIDS (Hospenthal *et al*., 2004). There are not many choices of antifungal drugs to treat the increasing occurrence of systemic fungal infections despite the introduction of new echinocandin class and addition of triazole antifungal drugs (Rogers, 2006).

Clotrimazole is an effective and safe therapy for the treatment of resistant esophageal candidiasis (Ginsburg *et al*., 1981; Collins & Van Sickels, 1983). Besides that, fluconazole was reported to be very effective in the treatment of oropharyngeal candidiasis (Koks *et al*., 2002). Apart from that, ketoconazole is an effective oral treatment for vaginal candidiasis (Bisschop *et al*., 1979). Ketoconazole was also reported to be effective in the treatment of skin, nail bed and buccal candidiasis among immunocompromised individuals (Horsburgh Jr & Kirkpatrick, 1983). Intravenous miconazole was used to treat systemic candidiasis in a pair of Siamese twins and a premature newborn. The condition of patients in both cases were considered to be life-threatening. However, they responded well to the treatment and recovered. Therefore, miconazole can be an alternative to amphotericin B for the treatment of systemic candidiasis in neonates (Sung *et al*., 1979). The management of candidiasis consists of drug therapy and other measures to treat underlying causes (Hay, 1999).

The therapeutic regimen of azole drugs is not effective due to malabsorption, interference due to low gastric pH (ketoconazole, itraconazole), interaction with other drugs which include rifampicin (itraconazole, fluconazole, ketoconazole) and the emergence of resistant strains of *C. albicans*, *Candida krusei* and *Candida glabarata* in recent years (Hoepelman & Dupont, 1996). Echinocandin drugs have become an important cure for the treatment of invasive candidiasis. In fact, echinocandin drugs can be considered as the first-line therapy for candidiasis patients. Anidulafungin, micafungin and caspofungin have also been proven effective (Kett & Cubillos, 2008). Apart from amphotericin B, fluconazole and itraconazole, clinicians can also select echinocandin class of antifungal agent, caspofungin and also the new broad spectrum of azole, voriconazole drug. Other options of azole and echinocandins are now being tested. Antifungal susceptibility testing plays an important role in the choice of antifungal therapy (Hospenthal *et al*., 2004). According to Gibbs (1998), fluconazole is effective and safe in the treatment of patients with systemic candidiasis.

The metabolism of fungal pathogens is altered in several ways when exposed to antifungal drugs. Initially, cells try to resist the inhibitory effect of antifungals by developing several resistance mechanisms. This enables the growth of cells at a higher concentration of antifungal agents compared to normally susceptible cells. When the antifungal drug has reached the concentration causing growth inhibition, the agent can be determined whether it is fungistatic or fungicidal (Sanglard, 2003).

2.3 Antifungal Resistance by *Candida* species

The emergence of *Candida* strains resistant to antifungal agents is commonly reported (Pfaller, 1995). The advancement of antifungal therapy can reduce the occurrence of candidiasis in some parts of the world but it may also cause the emergence of resistant strains in other areas. Resistance to azole drugs is a major concern in the treatment of candidiasis. During the initial development of antifungal therapy, the only drug reported to be resistant to strains of *C. albicans* is flucytosine. However, resistance to azole drugs has been reported in long-term therapy with ketoconazole for the treatment of chronic mucocutaneous candidiasis. Another important concern regarding drug resistance is the problem of cross-resistance (Hay, 1999). The extensive use of fluconazole has resulted in treatment failures. Resistance is particularly observed with relapses of oropharyngeal candidiasis among AIDs patients and other conditions (Lupetti *et al*., 2002).

Cases of thrush that were not responsive to fluconazole or requiring higher doses began to be reported. The resistance of *Candida krusei* to azole antifungals and reduced susceptibility of *Candida glabarata* were indicated during the early stages of the study (Hospenthal *et al*., 2004). Strains of *C. albicans* demonstrated resistance to fluconazole and also cross-resistance to other triazoles. Nystatin, boric acid or flucytosine can be used for treating azole resistant infections. Compliant patients' failure to respond to therapy and persistent clinical findings of positive yeast culture are indication of antifungal resistance (Sobel *et al*., 1998). This acquired resistance frequently occurs in species other than *C. albicans*. *Candida glabarata* is the second

most common cause of invasive candidiasis and has reduced susceptibility to fluconazole compared to *C. albicans* (Rogers, 2006).

2.4 Alternative Therapy

Progress of medical sciences comprising the latest techniques such as microsurgery, organ transplant, radio and chemotherapy and artificial organs that enable fast recovery from diseases for the well-being of humans. Although scientific medicine has managed to penetrate many parts of the world, its ultimate satisfaction is yet to be guaranteed. This is due to the undesirable side effects and suffering incurred due to drug administrations such as allergies or hypersensitivities which are sometimes life-threatening or can result in permanent disability. This is also due to the fact that some rural areas do not have advanced medical facilities. There are two types of medical systems, namely cosmopolitan medicine and indigenous medicine. Indigenous medicine has been used as an alternative to scientific medicine and therefore, is known as alternative medicine. Alternative medicine refers to those medicine other than modern scientific medicine. History has proven and will continue to show evidence that alternative medicine and complementary medicine are complementary. Both have their advantages and play important roles in promoting the well-being of mankind (Jingfeng, 1987). The application of alternative medicine can reduce the cost of expensive and highly complex health care of modern medical science (Aakster, 1986).

2.5 Lactoferrin

Lactoferrin is a 78-80 kDa molecule present in most of the exocrine secretions such as milk, colostrum, serum, semen and tears. Hence, being part of the body it does not induce any treatment side effects (Kanyshkova *et al*., 2001). Lactoferrin exerts a broad range of antimicrobial properties against various bacteria, virus and fungi *in vitro*. Human and bovine lactoferrin have 69% homologuous in their sequences and structurely very similar as observed at the tertiary level (Pierce *et al*., 1991). Lactoferrin contributes its antimicrobial properties as an intact molecule, monoferric lobes and active peptides as part of the host defense against microbial diseases. Lactoferrin is rich in cationic and hydrophobic antimicrobial peptides which could be targeted against microorganisms. These peptides are conserved within the polypeptide chain of lactoferrin and are released by proteolytic enzyme digestion. Although many antimicrobial peptides from lactoferrin have been found, only three have been thoroughly studied. These peptides are LF1-11, lactoferrampin and lactoferricin (Figure 2.1). Peptides play an important function in the antimicrobial properties of lactoferrin. The antimicrobial properties of cationic peptides is because of cytoplasmic membrane disruption of target cells. The presence of antimicrobial peptides in one domain of lactoferrin shows that the protein causes disruption of membrane integrity which confers its antimicrobial activity (Sinha *et al*., 2013).

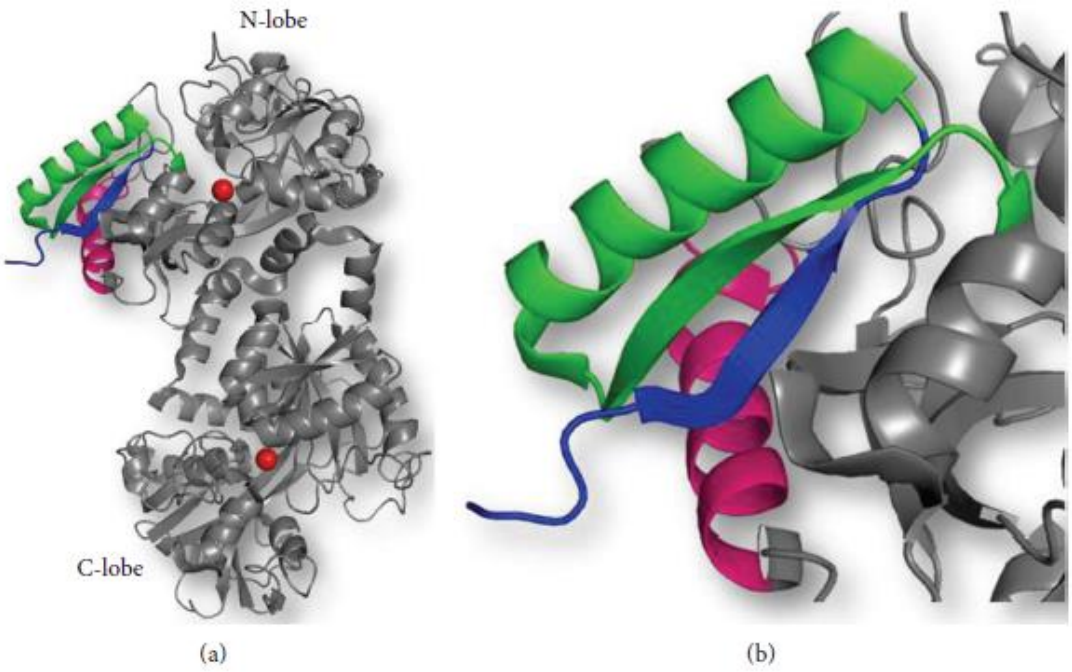

Figure 2.1: (a) Structure of Lactoferrin Indicating Positions of LF1-11 (blue), Lactoferrampin (pink), and Lactoferricin (green) Peptides in the N terminal lobe. (b) The Magnified Structure Demonstrating the Position of Peptides in Detail

Source: (Sinha *et al*., 2013)

2.6 Nanotechnology

Nanotechnology provides advantages such as slow or sustained release, target specificity, better efficiency, less side effects and enhance the rate of drug delivery (Kanwar *et al*., 2011). Drugs or biomolecules are loaded onto nanocarriers made of biocompatible and biodegradable substances such as synthetic proteins, peptides, polysaccharides, lipids, nucleotide and biodegradable polymers. Latest research in pharmaceutical has caused the development of proteins and peptides for the application in the treatment of diseases. Insulin is a protein employed for the

treatment of diabetes (Kanwar *et al*., 2011). Nanoparticles are very useful at the molecular level due to the high surface area and the ability to be customized. This include the design of nanoparticles to target specific cells and actives sites. Due to their small size, nanoparticles are able to pass through gaps that seem cannot be penetrated solving the problems relating with oral drug administration. The term "nanoparticles" refer to various structures. A few examples that are being studied for delivery of drugs include dendrimers, nanocarriers, nanotubes and degradable polymer particles. The most crucial property of nanoparticles is the size and size distribution. The size of nanoparticles affects the stability of particles, cell uptake, rate of drug release, rate of degradation, toxicity and ability of targeting cells (Singha & Lilliard, 2009). The small diameter of nanocapsules provides a high ratio of surface area to volume which can drastically elevate the bioavailability of any nanoformulated drug or protein (Shaikha *et al*., 2009).

2.6.1 Alginate-Enclosed Chitosan-Calcium Phosphate-Loaded Ferum-Bovine Lactoferrin Nanocapsules (NCs)

Calcium phosphate nanocapsules (NCs) replenish the calcium content which is necessary for a healthy body (Kanwar *et al*., 2012) and has very good biocompatibility. Chitosan is a mucoadhesive polymer which has health benefits (Gades & Stern, 2005). Due to its mucoadhesive characteristics, it is attracted to the internal surface of the digestive tract causing delivery directly to the walls decreasing contact to the intestinal fluids which enhances the delivery of the nanoformulated drug or protein (Kawashima *et al*., 1999). The US Food and Drug Administration has

declared sodium alginate as safe for oral intake (Tugcu-Demiroz, 2007). Alginate and chitosan are polymers which are biodegradable and calcium phosphate is naturally occuring in teeth and hence are safe to be utilized in NCs. Bovine lactoferrin is loaded onto NCs to protect it from degradation of the gut. As such, the bioavailability of lactoferrin at the site of infection does not change and has demonstrated optimum efficacy (Kanwar *et al*., 2008, Kanwar *et al*., 2012; Kanwar *et al*., 2015). Hence, NCs composed of three layers which are calcium phosphate ferum bovine lactoferrin nanocore followed by chitosan coating and then further coated with alginate. The alginate coating is to protect the ferum bovine lactoferrin from degradation in the stomach acidic condition, considering that alginate in the un-ionized form only allows diffusion of the protein in a slow rate in a lower pH environment (Zhang *et al*., 2008). In the intestine, the alginate layer is shed off and the NCs are absorbed by cells in the intestine. Iron-saturated ferum bovine lactoferrin is loaded on the calcium phosphate ceramic nanocore and further coated with chitosan and alginate (Figure 2.2A and 2.2B). Spherical size with particle size between 300 to 350nm is shown by transmission electron microscope (Figure 2.2C) and scanning electron microscopy images (Figure 2.2D). The average size of 205$\pm$15nm for calcium-coated calcium phosphate (CSC) inner core was demonstrated that was increased to 322$\pm$27.2 following coating with alginate to develop the final form of alginate-enclosed chitosan-coated calcium phosphate (ACSC).

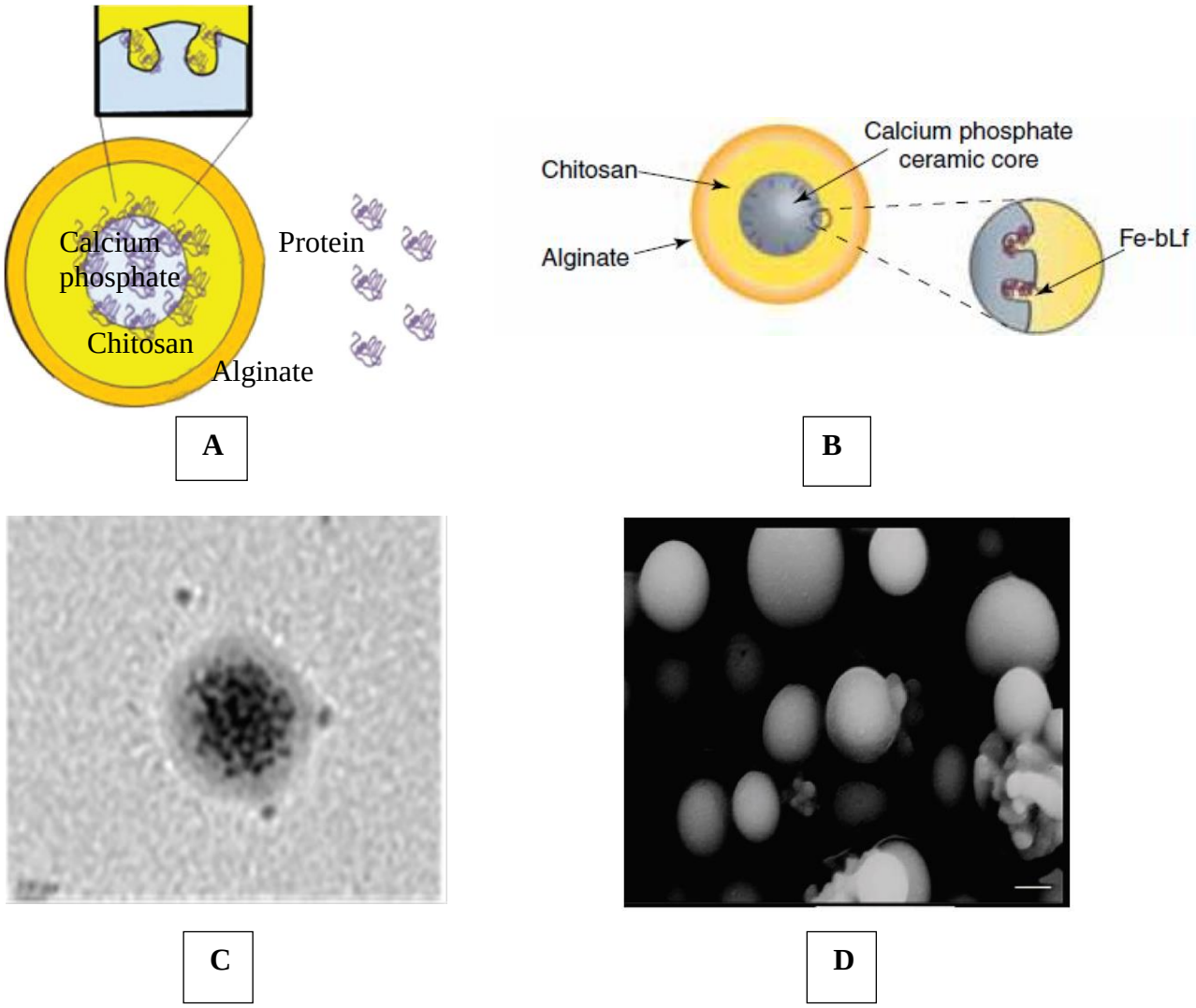

Figure 2.2 : Structural components of NCs

(A) & (B) NCs have spherical morphology under, (C) transmission electron microscopy, (D) scanning electron microscopy

Source: (Mahidhara *et al*., 2015)

The stability and size of NCs were studies over a range of pH(2-8) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and dynamic light

scattering (Mahidhara *et al*., 2015). NCs demonstrated little degradation at pH2 by dynamic light scattering. Only 15% of NCs demonstrated degradation while 85% were intact with the size of 300-330 nm. Therefore, their study showed the stability of NCs at acidic pH. However, the size of NCs was reduced to 205 nm due to shedding of the alginate layer. Their findings showed that an outer coating of alginate can protect a large portion (85%) of NCs from acid degradation at pH2, that is utilized to mimic the physiological (acidic) condition in the stomach of mice. The purpose of using alginate layer is to protect the inner core from degradation in the gastrointestinal system and alginate itself is fragmented in the process releasing the chitosan-coated calcium phosphate inner core. Therefore, the amount of chitosan-coated calcium phosphate NCs that have reached the final destination will be abundant .

2.7 Antimicrobial Peptide

Antimicrobial peptides (AMPs) are a diverse class of small proteins (12 to 50 amino acids) that are associated together due to their innate antimicrobial properties. Antimicrobial peptides have been discovered several decades ago, but it is only recently that their function is emphasized as vital to mammalian immune response (Gallo *et al*., 2002). Antimicrobial peptides are involved in the innate immune response and are used as the first line of defense by many organisms such as plants, insects, bacteria and vertebrates. Since antimicrobial peptides play a role as effectors in the innate immune system, they directly kill a broad range of microorganisms such as gram-positive and gram-negative bacteria, fungi and certain viruses. Apart from

that, these peptides interact with the host that cause events that complement their function as antibiotics. Although important, less emphasis has been put on antimicrobial peptides due to its underestimated role in human immunology.

Generally, antimicrobial peptides can be categorized into a few categories on the basis of their structures but most of them have a few similar structural features which include a cationic charge and the ability to interact with bacterial membranes through hydrophobic amino acids. Based on these common characteristics, two main families of antimicrobial peptides have been characterized, which are defensins and cathelicidins (Gallo *et al*., 2002). These forms have assisted in determining the importance of charge and hydrophobicity as the most vital properties responsible for antimicrobial properties (Rotem & Mor, 2009). Antimicrobial peptides are widespread in the animal and plant kingdom and play vital roles in defense mechanism. There are many varieties of antimicrobial peptides discovered and therefore, it is difficult to classify them except on the basis of their secondary structure (Zasloff, 2002).

The clinical developments between diseases and the production of antimicrobial peptides indicate that these peptides have vital and various functions (Gallo *et al*., 2002). Antimicrobial peptides are natural occurring antibiotics. As these peptides are genetically encoded and the rate of antibiotic resistance is much greater compared to mutations in mammals, a question has arisen as to why there is no emergence of antibiotic resistance towards peptides. There are various possibilities that explain this phenomenon. One of the possibilities is that the mechanism of

action of antimicrobial peptides which usually involves the penetration into the microbial membrane is considered a niche to the target microorganisms. Therefore, the cost for the emergence of resistance outweighs the need to maintain the growth of membrane integrity. Another reason for the persistence of antimicrobial properties is the combination of the antibiotic functions of peptides and their role as soluble immune mediators. Defensins and cathelicidins are chemotactic.

The unique properties of antimicrobial peptides in defense of microorganisms and the rise of antibiotic resistance to conventional antibiotics have led to studies in the development of antimicrobial peptides. Some of the clinical uses include infected diabetic foot ulcers, oral mucositis, meningococcal meningitis, catheter infections, acne, cardiac ischemia and fungal infections. Topical applications are likely to have the most immediate uses. Clinical studies of these antimicrobial peptides has potential benefits in the treatment of inflammatory and infectious diseases (Gallo *et al*., 2002). Antimicrobial peptides are currently employed as antimicrobial alternatives to chemical food preservatives and are also commonly used as antibiotics. Besides that, they also have potential applications in the pharmaceutical and food additive industry (Li *et al*., 2012).

2.8 Milk Derived Antimicrobial Peptides

HLopt2 (a modified peptide sequence based on amino acid residues 20-31 of the N-terminal end of human lactoferrin (hLF) has more potent antifungal properties

compared to hLF against all *C. albicans* and *Candida neoformans* except for *Candida glabarata* (Kondori *et al*., 2011). Human lactoferrin is derived from breast milk and HLopt2 was synthesized by an automated peptide synthesizer. A percentage of propidium iodide positive cells was used to demonstrate permeability of cytoplasmic membrane of *C. albicans* ATCC 64549 incubated with peptides for two hours. In another study, treatment of *C. albicans* and *Candida neoformans* with HLopt2 altered the surface of yeast significantly whereby the surface of *C. albicans* seemed punctured with deep pits and blebs were observed throughout the surface of the yeast. In that study, rougher surfaces were observed for *Candida neoformans* and leakage of substances were detected between the cells and the surface was covered heavily with blebs. Based on that study, treatment with HLopt2 caused greater than 99.9% death of yeast cells. In that study, following the two hours treatment of the yeast cells with hLF, agglutination of the cells were seen and surface changes of the cells were noticed. A more wrinkled cell surface was observed compared to the untreated cells. In addition, they showed that HLopt2 and HLBD1 demonstrated *in vivo* anticandidal activity in infected mice and there was a significant reduction in colony-forming units in the washings from the *Candida* infected skin in mice at a dose of 400 µg/spot by both peptides. The mode of action of peptides in that study was the disorganization of the cell wall and disruptions of the cell membrane and mitochondrial membrane (Kondori *et al*., 2011).

In a previous study, the MIC of lactofericcin B, a peptide derived from the enzymatic cleavage of bovine lactoferrin was evaluated against nine strains of *C. albicans* (Bellamy *et al*., 1993). *C. albicans* was inhibited by lactofericcin B between 18 to 60 µg/mL (6 to 20 µM) in 1% peptone or PYG medium (Bellamy *et al*., 1993).

That study indicated that *C. albicans* were highly susceptible to lactofericcin B. Comparatively, the MIC of bovine apo-lactoferrin against the strain JCM1542T in 1% peptone obtained in that study was 2.0 mg/mL (25 μM) which was less effective than its active peptide. The antimicrobial properties against *C. albicans* decreased in the presence of potassium chloride (KCl) or magnesium chloride ($MgCl_2$) in the range of 25 to 100 mM or $CaCl_2$ in the range of 1 to 5 mM. Apart from that, lactofericcin B caused the colony-forming capabilities of *C. albicans* to be lost rapidly. The rate of killing of the yeast by lactoferricin B was both time and dose dependent. The findings showed that *C. albicans* were highly sensitive to suppresion by lactoferricin B indicated that active peptides of lactoferrin can be potential host defense against *C. albicans* infections.

The anticandidal mechanism of lactoferricin B was evaluated whether it has direct interaction with the cell surface by determining the cell-binding abilities of ^{14}C-labeled lactoferricin B (Bellamy *et al*., 1993). It was found that the peptide bound to *C. albicans* and maximum binding happened in 60 minutes under the set conditions of cells in 25 mM potassium phosphate buffer, pH 6.0 were treated with ^{14}C-labelled lactoferricin B at 30 ^{o}C. The rate of binding was found to be proportional to the rate of killing caused by lactoferricin B. It showed that the extent of binding was dose dependent and the number of molecules bound was about 2.3×10^8/cell at the highest peptide concentration of 150 μg/mL. This number for the binding to specific protein receptors is greater than expected indicating that this peptide can interact with repeated parts of the cell surface. The degree of binding decreased in the presence of Mg^{2+} and Ca^{2+} ions as both helped to reduce the anticandidal properties of lactoferricin B. The findings indicated that cell-binding

played an important function in the anticandidal action of lactoferricin B. Thus suggested the involvement of ionic interactions in cell-binding. The degree of binding was pH dependent and optimum binding was at pH 6.0. The killing effect of lactofericcin B was maximum at the optimum pH for cell binding. This further supports the finding that cell binding was vital for the lethal effect of *C. albicans*.

It was found that the peptide caused a dramatic alteration in cell morphology when the ultrastructural structures of the yeast were examined by transmission electron microscope. Organelles were no longer noticeable following 60 minutes of treatment while peptide debris in the cytoplasm gradually increased during the duration of treatment. This indicated that lactoferricin B may cause autolysis in *C. albicans*. Findings of their study exhibited a correlation between cell-binding and the killing effect of lactoferricin B against the yeast. Therefore, it can be concluded that direct interaction with the cell surface contributed to the anticandidal mechanism. The findings of their study that lactoferricin B caused a killing effect against *C. albicans* indicated that the structural component of lactoferrin responsible for anticandidal activities is the region corresponding to this active peptide (Bellamy *et al*., 1993).

Lactoferricin B (LF-B) demonstrated antifungal properties against several pathogenic yeasts and filamentous fungi. *Candida tropicalis*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans* and *Epidermophyton floccosum* demonstrated low MIC values (0.31 to 2.5 µg/mL). However, most strains of non-pigmented hyphomycetes and zygomycetes were resistant to LF-B (MIC > 80 g/mL).