SYNTHESISE AND CHARACTERIZATION OF POLYMERIC DERIVATIVES OF ISONIAZID AS ANTI-TUBERCULOSIS DRUGS

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excepts, equations, summaries and references, which have been duly acknowledged.

8th APRIL 2014

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CERTIFICATION

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ABSTRACT

Conventional method for treatment of tuberculosis requires efficient therapy systems due to inefficiency of drug administration and multi drug resistance. In recent years of many studies were carried out to improve drug delivery systems. Polymers are one of the most essential elements for drug delivery research due to their versatility. By the approach of multivalency, drug delivery studies gained new vision. Polymers with multiple site of attachment were used to create multivalent interactions. In this research, natural polymer, chitosan was used as polymeric drug carrier. Chitosan was initially modified to enhance it as an efficient drug carrier. Chitosan was etherified with monochloro acetic acid to produce carboxymethyl chitosan. N-acylation was carried out onto carboxymethyl chitosan to produce N-succinylcarboxymethyl chitosan. N-succinylcarboxymethyl chitosan was then coupled with a known anti-tuberculosis drug, isoniazid to produce multivalent isoniazid. A series of 12 compounds were successfully synthesized. The evaluations of chemical and physical properties of multivalent isoniazid compounds were conducted after purification by dialysis. Several factors affecting the synthesis of multivalent isoniazid such as temperature and reaction medium were studied. Among the 12 compounds compound C had highest degree of substitution value with 0.36 and it is considered as the most efficient drug substituted compound. The optimum reaction condition for producing compound C with DS value 0.36 was 50 ^{0}C with water and isopropanol at 2:8 reaction medium. Isoniazid and N-succinylcarboxymethyl chitosan interactions and changes in properties were determined by FTIR analysis. From FTIR spectroscopy analysis, the existence of isoniazid transmittance bands in synthesised multivalent compounds with slight changes indicated that isoniazid chemically interacted with NSCMCS. The SEM micrograph observation also reconfirmed the substitution process, the unmodified chitosan had smooth surface whereas rough features observed on the multivalent compounds. Particle size measurement revealed the synthesized compound has larger radius size than isoniazid. Compound C has the largest radius with 954.1 nm whereas the radius of isoniazid was 110.8 nm. Large size of compound has the ability to prolong the circulating duration. Drug stability profile of synthesized compounds was studied in pH buffer 3.5 and 7.4. Results revealed that the release of isoniazid was more stable in alkaline medium. This also indicated that isoniazid cleaved from N-succinylcarboxymethyl chitosan backbone through hydrolysis as it linked through amide. The solubility profile showed that the final compounds not soluble towards the water, however carboxymethyl chitosan and N-succinylcarboxymethyl chitosan does soluble in water. Finallyantimycobacterial assay was done against *M. tuberculosis* H37Rv. The results revealed that compound A was the most potent drug as it inhibits the *M. tuberculosis* with isoniazid concentration of 1.5822 mg/mL. The minimum inhibitory concentration value obtained was 0.0781 µg/mL.By this study, the efficiency of the multiplicity effect in drug delivery was shown.

ABSTRAK

SINTESIS UBAT POLIMER ANTI-BATUK KERING

Kaedah konvensional rawatan untuk batuk kering memerlukan system terapi berkesan kerana ketidakcekapan pentadbiran dadah dan pelbagai rintangan dadah. Kebelakangan ini banyak kajian yang dijalankan untuk meningkatkan system penyampaian ubat. Polimer adalah salah satu elemen yang paling penting untuk penyelidikan penghantaran dadah kerana fleksibiliti mereka. Dengan pendekatan multivalensi, kajian penyampaian ubat mendapat wawasan baru. Polimer dengan laman pelbagai tempat mengikat digunakan untuk mewujudkan interaksi multivalensi. Dalam kajian ini, polimersemulajadi, chitosan telah digunakan sebagai pembawa ubatpolimer. Kitosan pada mulanya diubahsuai untuk meningkatkan ia sebagai pembawa ubat cekap. Kitosan telah 'etherified' dengan asidasetikterkloro untuk menghasilkan karboksimetilkitosan. Pengasilan-N telah dijalankan ke karboksimetilkitosan untuk menghasilkan 'N-succinylcarboxymethyl' kitosan. 'N-succinyl' carboxymethyl kitosan kemudian ditambah pula dengan ubat anti-batukkering, isoniazid untuk menghasilkan isoniazid multivalensi. Satusiri 12 sebatian telah berjaya disintesis. Penilaian sifat-sifat fizikal sebatian kimia isoniazid multivalensidan telah dijalankan selepas pembersihan oleh dialisis. Beberapa faktor yang mempengaruhi sintesis isoniazid multivalent seperti suhu dan medium tindak balas telah dikaji. Antara sebatian, sebatian C mempunyai tahap tertinggi nilai penggantian dengan 0.36 dan ia dianggap sebagai ubat yang paling berkesan digantikan majmuk. Keadaan optimum tindakbalas untuk menghasilkan sebatian C dengan DS nilai 0.36 ialah 50 ^{0}C dengan medium tindak balas yang mempunyai air dan 'isopropanol' berasaskan nisbah 2:8. Isoniazid dan 'N-succinylcarboxymethyl' chitosan interaksi dan perubahan dalam ciri-ciri telah ditentukan oleh analisis FTIR. Dari analisis spektroskopi FTIR, kewujudan kumpulan pemindahan isoniazid dalam sebatian disintesis multivalent dengan sedikit perubahan menunjukkan bahawa isoniazid berinteraksi kimia dengan 'N-succinylcarboxymethyl' chitosan. Pemerhatian mikrograf SEM juga menyahkan proses penggantian berlaku. Kitosan yang diubah suai mempunyai permukaan licin manakala ciri-ciri kasar diperhatikan pada sebatian multivalent. Ukuran saiz zarah mendedahkan kompaun disintesis mempunyai saiz lebih besar daripada isoniazid. Sebatian C mempunyai jejari terbesar dengan 954.1 nm manakala jejari isoniazid adalah 110.8 nm. Sebatian yang bersaiz besar mempunyai keupayaan untuk memanjangkan tempoh peredaran dalam badan. Profil kestabilan ubat sebatian disintesis telah dikaji dengan 'buffer' pH 3.5 dan 7.4. Hasil kajian menunjukkan bahawa pembebasan isoniazid lebih stabil dalam medium beralkali. Ini juga menunjukkan bahawa isoniazid melekang dari 'N-succinylcarboxymethyl' kitosan melalui hidrolisis kerana ia dihubungkan melalui 'amide'. Profil kelarutan menunjukkan bahawa sebatian terakhir tidak larut air ke arah itu, bagaimanapun 'carboxymethyl' kitosan dan 'N-succinylcarboxymethyl' kitosan larut dalam air. Akhirnya assay antimycobacterial dilakukan terhadap *M. tuberculosis* H37Rv. Keputusan menunjukkan bahawa sebatian A adalah ubat yang paling berpotensi kerana ia menghalang *M. Tuberculosis* dengan kepekatan isoniazid daripada 1.5822 mg / mL. Nilai kepekatan perencatan minimum yang diperoleh ialah 0.0781 µg / mL. Dengan kajian ini, keberkesanan kesan kepelbagaian dalam penyampaian ubat telah ditunjukkan.

TABLE OF CONTENTS

CHAPTER 3: METHODOLOGIES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

BCG	Bacillus Calmette-Guérin
CMCS	Carboxymethyl chitosan
DOTS	Directly observed treatment, short-course
DS	Degree of substitution
EMB	Ethambutol
ETA	Ethionamide
FT-IR	Fourier transform infrared spectroscopy
HIV	Human immunodeficiency virus
INH	Isoniazid
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
NSCMCS	N-succinylcarboxymethyl chitosan
RIF	Rifampicin
SEM	Scanning electron microscope
STR	Streptomycin
TB	Tuberculosis
TEMA	Tetrazolium bromide microtiter assay

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

1.1 Background

Tuberculosis (TB) is highly contagious bacterial infection which is caused by *Mycobacterium tuberculosis* (Nathanson *et al.,* 2010; Gupta *et al.,* 2007).It is a treatable airborne infectious disease thatkills almost 2 million people every year (Keshavjee *et al.,* 2012; Dube *et al.,* 2012). World Health Organization (WHO) report on 2012 stated that the targets of Millennium Development Goal (MDG) has achieved where the rate of TB new cases fell at a rate 2.2 % between 2010 and 2011. However, the global burden of TB remains enormous. In 2011, there were 8.7 million new cases with 1.4 million died from TB (WHO, 2012).

Scientists believed that TB disease has been around for over 20,000 years with the existence of Egyptian mummies and fromarchived centuries-old Indian and Greek scripts (Shet, 2012). In 1943, the first natural antibiotic compound was discovered (Sierra, 2006). However, the resistance to streptomycin was developed and soon necessitated by the need of the next generation of antibiotics against *M. tuberculosis* (Shakya *et al.,* 2012). Subsequently a series of antimycobacterial drugs have been discovered and classified them as first line and second line drugs (Cabrera-rivero *et al.*, 2012).

Globally, drug resistant TB has been recognized by WHO as the main obstacle in the fight against TB (Dube *et al.*, 2012). The resistance was developed against two most powerful first line drugs namely isoniazid and rifampicin (Sharma & Mohan, 2006). Resistance to anti TB drugs emerged as a serious phenomenon primarily due to several factors namely health mismanagement attention, inadequate therapy courses, antibiotic misuse, insufficient socioeconomic

conditions, presence of immunodeficiency disorders and low patient compliance (Bueno *et al.*, 2011; Jordao *et al.*, 2011; Ahsan *et al.*, 2011). Moreover co-infection of TB-HIV fuels the problem by increasing drug interaction which produces toxic side effects (Sierra, 2006). Antimycobacterial therapy is available for tuberculosis but the efficiency and the percentage of curable patients through the treatment is the challenge. It has been 40 years, yet there is still no new drug enter the market for TB. Thus the focus shift towards using innovative drug delivery strategies which might improvise anti-tubercular (anti-tb) chemotherapy efficacy, thereby enhancing patient compliance at once achieved the goal of the Global Plan to Stop TB, 2011 through 2015 (Faria *et al.*, 2012; Bueno *et al.,* 2010; Sharma *et al.*, 2006).

The importance of this research study is to create an effective and innovative drug delivery system against TB disease. This is because the first line drugs potencies and therapeutic effects are limited or reduced because of the partial degradation that occurs before they reach the desired target in the body. Hence polymers have been chosen as a vector or carrier to transport those drugs safely (Khandare *et al.*, 2006; Choi, 2004; Duncan, 2003; Cao *et al.*, 2005).

Polymers have been used extensively in drug delivery system as nanoparticles, microcapsules, laminates and matrices. In all these delivery systems, the drug is merely dispersed or incorporated into the system without the formation of covalent bonds between drug and polymer. However the originality of this study is to focus on a polymeric drug delivery system where the drug is covalently bonded to a polymeric backbone with multiplicity effect.

Polymers are able to create multiple binding sites where numerous drug molecules can attach to a single chain. The drugs bonded to multiple binding sites will be released at target site where accumulation of higher therapeutic effects will allow efficient inhibition of *Mycobacterium* infected cells. The approach of multivalent interaction will help in decreasing the drug resistant phenomena and might shorten the duration of TB treatment. This will save millions of life and have tremendous global benefits where a shorter TB regimen could improve treatment compliance. This shorter and simpler treatment will not only help cure those currently under the care but will also allow health workers to reach more people.

Among the polymer interested to be developed for biomedical and pharmaceutical applications are biopolymers because biopolymers presence abundance in nature, have good biocompatibility and have the ability to modify by simple chemistry (Sonia *et al.*, 2011). Chitosan is the only natural cationic polysaccharide and being explored for pharmaceutical drug delivery (Bernkop-Schnurch & Dünnhaupt, 2012). Chitosan gains interest due to the presence of amino group in the main backbone where most chemical modifications are performed (Bernkop-Schnurch & Dünnhaupt, 2012). Modification of chitosan does not change the fundamental skeleton but bring improved properties which widen its application. Furthermore the approval for human use by the US Food and Drug Administration (FDA) is an additional advantage (Ratna *et al.*, 2010; Terbojevich *et al.*, 2009).

1.2 Objectives of the Study

The objectives of this study were:

i. To synthesise multivalent isoniazid onto chitosan polymer.

ii. To characterize and determine the physical and chemical properties of the synthesized multivalent isoniazid.

iii. To determine Minimum Inhibitory Concentration (MIC) of the synthesized multivalent isoniazid against *Mycobacterium sp.*

1.3 Scope of the Study

In this study, chitosan biopolymer was used as a vector to carry a known anti-tuberculosis drug, isoniazid. The polymeric drug carrier builds with three parts namely a macromolecular carrier (chitosan), a cleavable linker and drug (isoniazid). The drug anchoring to polymeric drug carrier is via covalent bond. Twelve compounds were successfully synthesised through series of chemical reactions. These compounds were analyzed for the degree of substitution and solubility profile. The compounds were then characterized spectroscopically through infrared spectroscopy and particle size analyzer. The morphological character was determined using scanning electron microscopy. Thermal stability and character were analyzed using differential scanning calorimetry and thermo gravimetric analysis, whereas the stability behavior of the compounds through gastrointestinal

passage (mimic) also was also determined. Eventually the compounds were evaluated for antimycobacterial activity against *M. tuberculosis.*

1.4 Design and Synthesis of Polymeric Carriers

This study aimed to develop a polymeric drug delivery approach for delivery of chemotherapeutic drugs, isoniazid to cells infected by *Mycobacterium*. These polymeric drugs were synthesized via three steps of reaction namely carboxymethylation, succinylation and subsequent amidation of modified chitosan with amino groups of isoniazid. Chitosan modification was carried out to introduce a specific chemical substituent namely chloroacetic acid and succinic anhydride to alter the physical and chemical properties. Commonly the properties of chitosan modification defined by degree of substitution in which the average numbers of hydroxyl groups or amino groups were substituted in a glucose unit.

In order to produce an efficacious drug delivery carrier for TB treatment the drug carrier must have certain functional and structural properties. As such chemical modification of chitosan is necessary to improve the properties. Chitosan is known to be water insoluble as it has strong intermolecular hydrogen bonding which give the rigid crystalline structure. Several chemical modification required which this modification will aid in good solubility at once ensure a good connection with drugs.

Modification on the hydroxyl group is a promising candidate to improve the water solubility. Several research has been reported by (Zhu et al., 2005; Chen & Park, 2003; Zhu et al., 2007; An et al., 2009; Mourya et al., 2010) that amphilic properties of chitosan can be improved by the carboxymethyl chitosan. This is done by introducing monochloroacetic acid which is known as etherifying agent. This is an inexpensive method for chitosan modification. To proceed with this reaction, chitosan was activated by dissolving in alkaline solution. The concentration of sodium hydroxide used was 50 % which reported by Tokura and Chen co-workers as the optimum alkali concentration in carboxymethylation process (Tokura et al., 1984; Chen et al., 2003; Mourya et al., 2010). However the water soluble properties of the final compounds need to take into consideration as it is a three step reaction.

Preswollen of chitosan will disrupt the intermolecular hydrogen bonding where the rigid crystalline structure of chitosan extensively lost. This will give space to chloroacetic acid to penetrate into the polymer chains. The etherification process is known as Williomson's etherification. The reaction type involves is SN_2 or known as nucleophilic substitution. The hydroxyl group in chitosan is deprotonated to form alkoxide ion. The negative charge on the oxygen attacks chlorine (halide) which is very electronegative. However, SN_2 reaction is bimolecular which involves simultaneous bond making and bond breaking steps. The general mechanism involves is shown in Figure 1.1:

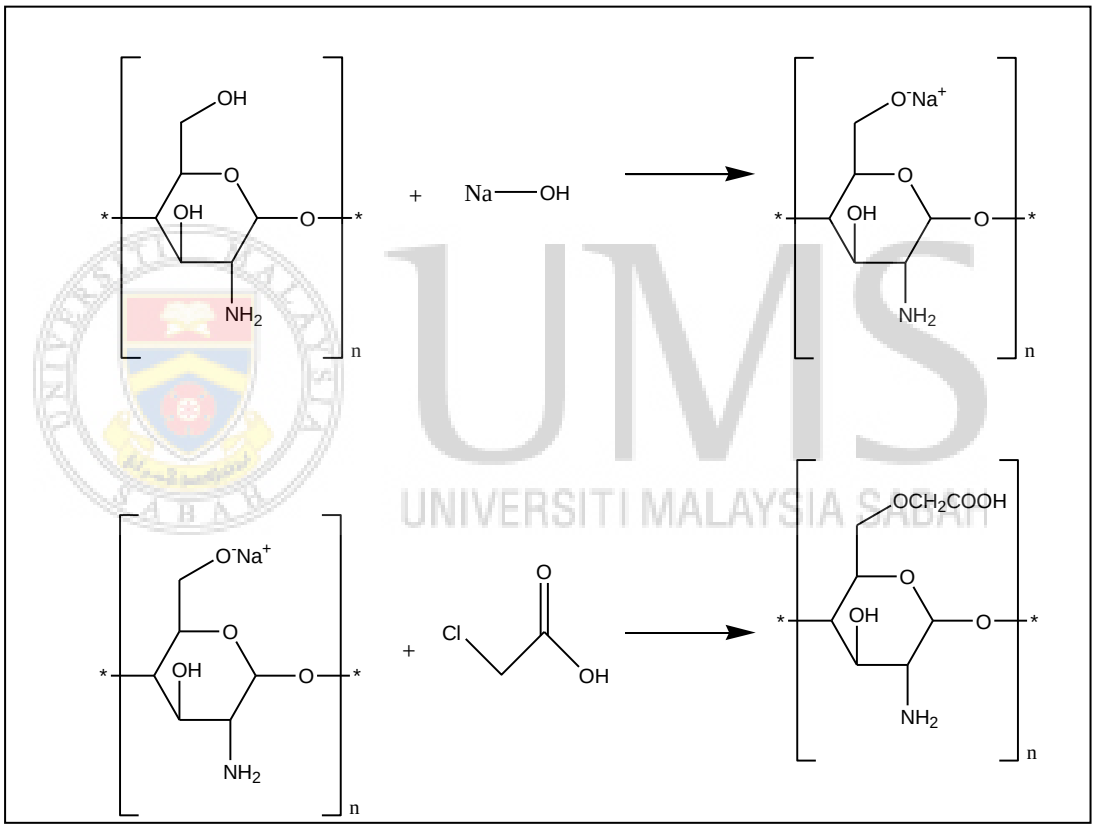


Figure 1.1: Reaction scheme of carboxymethyl chitosan.

After obtaining carboxymethyl chitosan, succinic anhydride can be introduced to the N-terminal of the glucosamine unit of chitosan. The mechanism of N-acylation is shown in Figure 1.2.

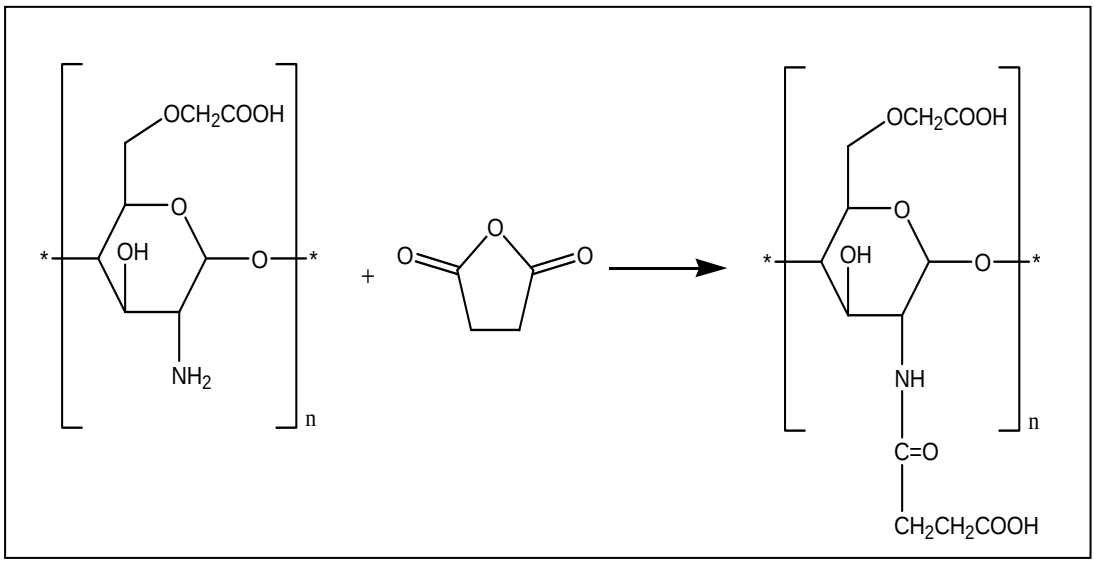


Figure 1.2: Reaction scheme of N-succinylcarboxymethyl Chitosan.

The successfully synthesized modified chitosan biopolymer will be then reacted with isoniazid. Prior to the reaction, the carboxyl group present in N-succinylcarboxymethyl chitosan need to be activated. Carboxylic acids need to be converted into carboxamides by treating them with amines. However, the reaction does not proceed rapidly. Initially, the amine (base) is protonated by carboxylic acids yielding an ammonium carboxylate. The salt is then heated to remove water. Eventually, carboxamide is formed. But water is a poor leaving group thus several reagents needed to make carboxylic acid's hydroxyl oxygen into the more efficient leaving group. The reagents are namely dihexylcarbodiimide (DCC) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodi- imide hydrochloride (EDC). EDC is preferred for the activation purpose as it is easily soluble and stable in water. The carboxylic acid's hydroxyl group converted into O-acylisourea ester, which is an effective leaving group. Hence the EDC activated carboxylic acid nucleophilically attacked by amino of isoniazid to form stable amide linkage (Patel *et al.,* 2010). The reaction mechanism of activation of carboxylic acid is shown in Figure 1.3.

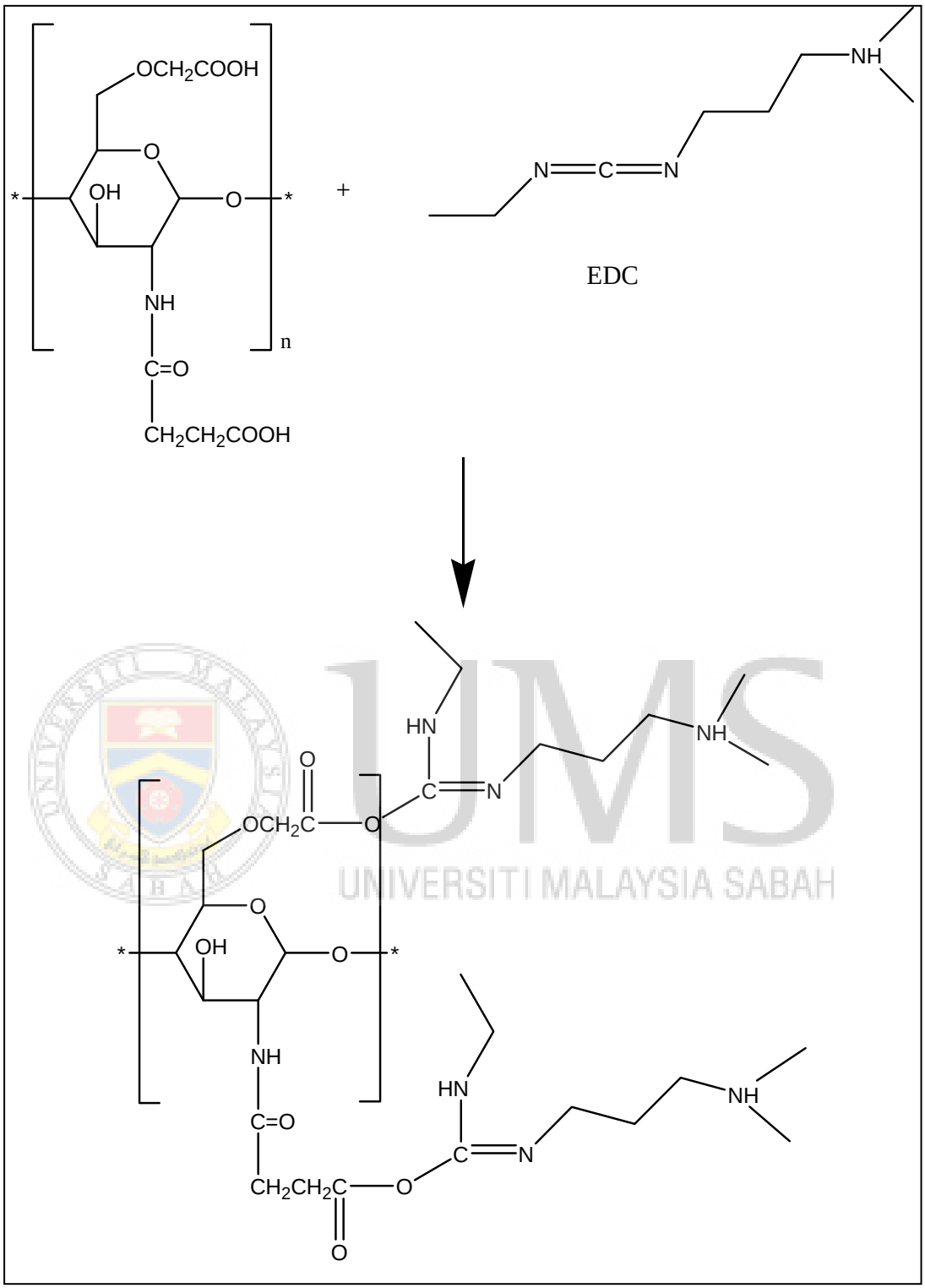
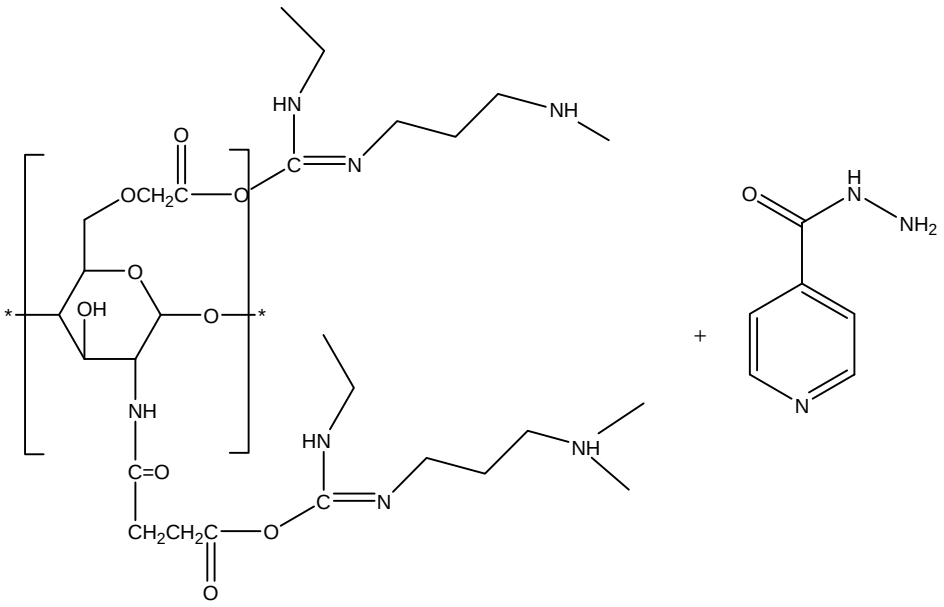


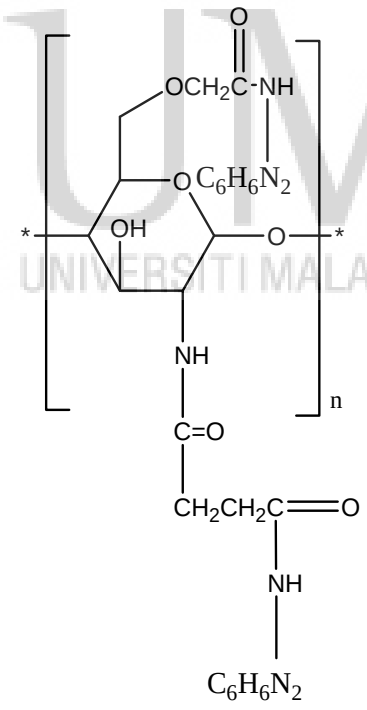
Figure 1.3: Reaction scheme of activation of carboxylic acid.

Formation of amide linkage between activated carboxylic acid of N-succinylcarboxymethyl chitosan and isoniazid shown in Figure 1.4:



activated N-succinyl carboxymethyl chitosan

isoniazid



multivalent isoniazid

Figure 1.4: Reaction scheme between N-succinylcarboxymethyl chitosan and isoniazid.

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is a disease scraping the human kind for millennia. It is microbial disease caused by *Mycobacterium tuberculosis* (Nathanson *et al.,* 2010; Gupta *et al.,* 2007).In 1993, the World Health Organization (WHO) declared TB to be a global public health emergency (Ramappa & Aithal, 2013; WHO, 2012). There are more TB cases in the world where it affects the countries which are not welll equipped to meet the demand (TB Alliance, 2011; Claire *et al.,* 2006). There were estimated 8.8 million incident cases of TB (range 8.5 million–9.2 million) globally in 2010, 1.1 million deaths (range, 0.9 million–1.2 million) among HIV-negative cases of TB and an additional 0.35 million deaths (range 0.32 million–0.39 million) among people who were HIV-positive (Behera, 2010). Most cases of tuberculosis evolved around South East Asia, Africa and Western Pacific regions (Lamrabet & Drancourt, 2012; WHO, 2010). However, Global Tuberculosis report by WHO in 2012 indicate that new cases of TB had fallen at a rate 2.2 % between the years 2010 to 2011. The TB mortality rate has decreased 41 % since 1990 (WHO, 2012).

Similar to other developing countries, TB is still apublic health problem in Malaysia despite preventiveand control measures were taken. TB is the top five diseases in Malaysia that lead to mortality (Rundi, 2010; Venugopalan, 2004). The incidence rate in Malaysia has been stagnant at around 58.7 to 65.6 per 100,000 populations in the last ten years. Theabsolute number of new cases has been increasing fromabout 15,000 new cases in 2002 up to 16,665 in 2006 (WHO, 2010; Rundi, 2010; Iyawoo, 2004). Sabah contributes one-third of the total cases in the country and has a notification rate forall cases of 100-200 per 100,000 people for

almost a decade now. Figure 2.1 shows an estimated TB incidence in the world in 2011.

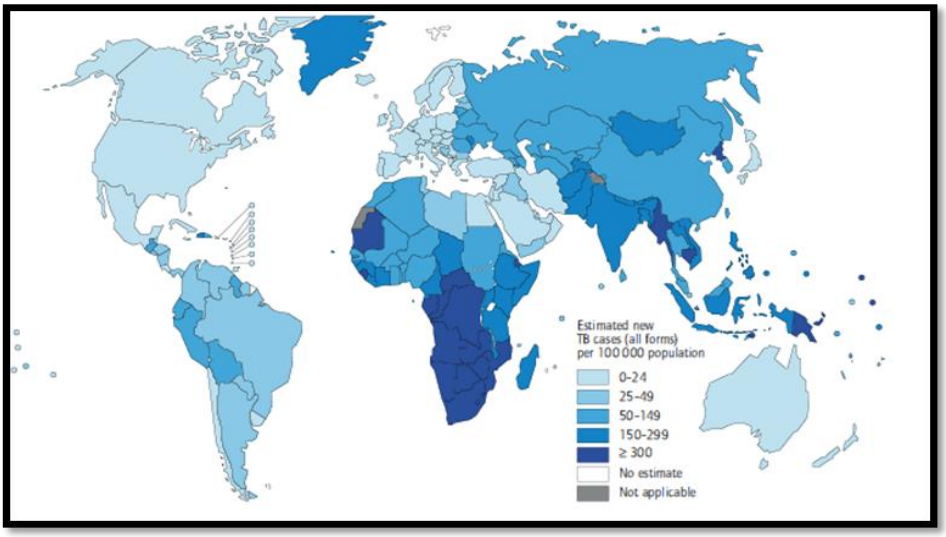


Figure 2.1: Estimated TB incidences in year 2011 (WHO, 2012).

One of the greatest challenges to fight with TB is Human Immunodeficiency Virus (HIV). A person infected with tubercle bacillus is at risk during developing active TB in their lifetime if the immune system is not impaired. HIV/AIDS fuels the tuberculosis epidemics in many ways, suchas promoting progression to active tuberculosis, increasing the risk of reactivation of latent tuberculosis infection, as well as increasing chances of tuberculosis infection once exposed to tubercle bacillus (Sierra, 2006; Paul *et al.,* 2005).

Furthermore, the emergence of multi drug resistant (MDR) and extensively drug resistant (XDR) strains of *M.tuberculosis* raises the mortality rates of human population. WHO defines MDR-TB as the bacteria become resistant to two first line drugs namely, isoniazid and rifampicin (Sharma & Mohan, 2006). MDR-TB caused by inadequate regimens by health care providers, patients' inadequate drug therapy, inadequate quality or supply of drugs, socioeconomic conditions with immunodeficiency disorders lead to the alarming emergence of multi drug resistant strains. The most prominent MDR TB cases are from India, China, South Africa, Nigeria and Indonesia where the mortality rate is at stagnant phase (Dube *et al.,*