SYNTHESIS, CHARACTERIZATION AND PROPERTIES OF CHITOSAN-POLY(METHACRYLIC ACID-CO-N-ISOPROPYLACRYLAMIDE) HYDROGEL FOR DRUG DELIVERY

SITI ZALIFAH BINTI MD RASIB

UNIVERSITI SAINS MALAYSIA

2019

SYNTHESIS, CHARACTERIZATION AND PROPERTIES OF CHITOSAN-POLY(METHACRYLIC ACID-CO-N-ISOPROPYLACRYLAMIDE) HYDROGEL FOR DRUG DELIVERY

by

SITI ZALIFAH BINTI MD RASIB

Thesis submitted in fulfilment of the
requirement for the degree of
Doctor of Philosophy

May 2019

ACKNOWLEDGEMENTS

Alhamdulillah. I am grateful to Allah SWT for giving me strength and guided me to complete this thesis. There are many people I would like to acknowledge here for their support, help, and encouragement along my journey in completing this thesis.

First of all, I would like to express my special thanks of gratitude to University Sains Malaysia especially to School of Materials and Mineral Resources Engineering lead by Associate Professor Ir. Dr. Syed Fuad b Saiyid Hashim with former dean Professor Dr. Zuhailawati binti Hussain for offering this great opportunity and providing enough resources, facilities, and instruments.

Furthermore, I would like to express my utmost gratitude and appreciation to my supervisor Professor Dr. Hazizan bin Md. Akil for his counsel, encourage and support throughout this research. I especially appreciate his theoretical insight, provide guidance and gave me freedom to make my own decisions on gaining independence in handling my research and completing this thesis. Hopefully this great experience worthwhile for my future.

I am deeply indebted to my co-supervisor, Dr. Zuratul Ain Bt. Abdul Hamid, who kept an eye on the progress of my work, provided her advice, and guidance from the early stage of this research as well. I would like to say thank you to lecturer, staffs, and technicians for their supporting, contribution and times. I would like to acknowledge Dr. Abbas Khan for all his support and motivation from the initial days of my study, until the moment I am completing this thesis.

I am very grateful with special thanks to my husband Mohd Hafiz bin Zamri who has been a constant source of support and encouragement. Unforgettable appreciation also to my son Firas bin Mohd Hafiz and my daughter Syaurah binti Mohd Hafiz for being the ultimate reason in finishing this thesis. Looking at them also always made me stronger, better and more fulfilled than I could have ever imagined. My deepest gratitude goes to my mom, Napipah binti Bahar. She is the one that always encourages me to keep continue my study. Also, thank you to my siblings for their unflagging love and continuous support.

Finally, thank you to my fellow lab mates for the stimulating discussions and critical advice, a great teamwork who always being there and bearing me the good and bad times during my wonderful days of my study.

TABLE OF CONTENTS

CHAPTER FOUR: RESULTS AND DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

IPN	interpenetrating polymer network
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
APS	Ammonium persulfate
Chitosan-p(MAA-co-NIPAM)	Chitosan-poly(Methacrylic acid-co- N-isopropylacrylamide
COO-	Carboxylate ion
-COOH	Carboxyl group
DI	Distilled water
DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified Eagle medium
DOE	Design of experiment
EPR	Permeability And Retention
FESEM	Field Emission Scanning Electron Microscope
FTIR	Fourier Transforms Infrared
L929	Normal fibroblast cell line
LCST	Lower critical solution temperature
MAA	Methacrylic acid
MBA	N,N'-methylenebisacrylamide
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
N_2	Nitrogen
$–NH_2$	Amino group

$-NH_3^+$	Ammonium ion
NIPAM	N-isopropylacrylamide
–OH	Hydroxyl group
PAA	Poly(acrylic acid)
PAAm	Poly(acrylamide)
PBS	Phosphate Buffer Solution
PDEAEMA	Poly(diethylaminoethyl methacrylate)
PDT	Photodynamic therapy
PEG	Poly(ethylene glycol)
pH/T-responsive	pH- and tempearature responsive
pKa	Log of acid dissociation constant
pKb	Log of basic dissociation constant
PMAA	Poly(methacrylic acid)
RES	Reticuloendothelial system
Rif	Rifampicin
SEP	Surfactant Emulsion Polymerization
SFEP	Surfactant-free Emulsion Polymerization
SIF	Intestinal fluids
$-SO_3H$	Sulfonic group
UV-Vis	Ultraviolet–visible
VPTT	Volume Phase Transition Temperature

LIST OF SYMBOLS

C_1	Concentration Rif without loading
C_2	Concentration Rif after loading
%	Percent
°	Degree
℃	Degree Celcius
F	Expression of kinetic model
g	Gram
KHz	Kilohertz
log	Logaritme
M	Molar
mg	Miligram
MHz	Megahertz
ml	Mililiter
mM	Milimolar
mV	millivolt
n	Release exponent
nm	Nanometer
r^2	Square correlation coefficient
rpm	Revolution per minute
t	Time
W_o	Initial weight
W_t	Weight of hydrogel after time, t

α	Significant level
μm	Micrometer

SINTESIS, PENCIRIAN DAN SIFAT-SIFAT HIDROGEL KITOSAN-POLI(ASID METAKRILIK-KO-N-ISOPROPILAKRILAMIDA) UNTUK PENYAMPAIAN UBAT

ABSTRAK

Penggunaan kitosan semakin mendapat perhatian didalam bidang perubatan terutamanya bagi aplikasi penyampaian ubat. Dalam kajian ini, kitosan telah dihasilkan bersama dua monomer iaitu asid metakrilik (MAA) and N-isopropilakrilamida (NIPAM) melalui proses pempolimeran emulsi membentuk hidrogel kitosan-poli(asid metakrilik-ko-N-isopropilakrilamida) [kitosan-p(MAA-ko-NIPAM] sebagai penyampai ubat. Pencirian hidrogel dilakukan dengan menggunakan Spektroskopi Inframerah Transformasi Fourier (FTIR), ^{13}C Resonans Magnet Nukleus (^{13}C NMR), Mikroskop Elektron Imbasan Pancaran Medan (FESEM), analisis saiz partikel dan Serakan Cahaya Dinamik (DLS). Kesan tindak balas terhadap rangsangan (pH dan suhu), hidrolisis, pemasukan ubat, pelepasan ubat dan ketoksikan telah dikaji. Hasil kajian mendapati bahawa hidrogel kitosan-p(MAA-co-NIPAM) yang dihasilkan menggunakan surfaktan (Span 80) berjaya mencapai saiz partikel kurang daripada 500 nm. Cas permukaan kationik hidrogel yang telah dihasilkan melepasi keperluan kestabilan bagi mengelakkan pengagregatan dalam sistem darah di atas 30 mV, pada pH 3 hingga 5 dan berpotensi meningkatkan penyampaian ubat melalui internalisasi selular sehingga pH 6. Ujian penyampaian ubat Rifampisin yang dikawal oleh gabungan penyebaran dan pembengkakan telah berjaya dipanjangkan untuk tempoh masa sehingga 72 jam dan ini membuktikan hidrogel bertindak balas terhadap rangsangan pH dan suhu persekitaran. Sel berdaya tinggi dari penilaian ketoksikan pula telah menunjukkan bahawa hidrogel adalah bahan bio yang mempunyai sifat

mesra manusia/haiwan. Oleh itu, dapatlah disimpulkan bahawa hidrogel kitosan-p(MAA-co-NIPAM) adalah layak sebagai pengangkut untuk penyampai ubat yang boleh dikawal.

SYNTHESIS, CHARACTERIZATION AND PROPERTIES OF CHITOSAN-POLY(METHACRYLIC ACID-CO-N-ISOPROPYLACRYLAMIDE) HYDROGEL FOR DRUG DELIVERY

ABSTRACT

The application of chitosan has received enormous attention in medical field particularly in drug delivery. In this study, chitosan with two monomers, methacrylic acid (MAA) and N-isopropylacrylamide (NIPAM) were prepared by emulsion polymerization to form a chitosan-poly(methacrylic acid-co-N-isopropylacrylamide) (chitosan-p(MAA-co-NIPAM)) hydrogel as a drug carrier. Hydrogels were characterized using Fourier Transform Infrared (FTIR) Spectroscopy, Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR), Field Emission Scanning Electron Microscope (FESEM), particle size analysis and Dynamic Light Scattering (DLS). The effect of stimuli responsive (pH and temperature), hydrolysis, drug loading, in-vitro drug release and cytotoxicity was investigated. The finding found that chitosan-p(MAA-co-NIPAM) hydrogel synthesized using surfactant (Span 80) successfully achieved particle size less than 500 nm. The cationic surface charge of synthesis hydrogel passed the stability requirement to avoid aggregation in blood system above 30 mV at pH 3 to 5 and potentially enhanced cellular internalization drug delivery up to pH 6. The release of Rifampicin drug was controlled by combination of diffusion and swelling as the drug released was prolonged to 72 hours indicating that the hydrogel responded to the pH and temperature environment. High viability cell from cytotoxicity assessment demonstrated that the hydrogels are human/animal friendly biomaterials. Thus, it can be concluded that chitosan-p(MAA-co-NIPAM) hydrogels are feasible as controlled drug delivery carrier.

CHAPTER ONE

INTRODUCTION

1.1 Background

Hydrogels are unique materials that can absorb water as a response to either a chemical, physical, or biochemical environment (Alpesh and Kibret, 2011; Ashraf et al., 2017; Constantin et al., 2017; Ullah et al., 2016). Hydrogel synthesis by hydrophilic polymeric materials revolutionised the hydrogel field because of its unique capability to look like actual tissue cells. By simple preparation methods such as chemical, physical, or both methods with different polymeric materials, it has been established that hydrogel is the most potential drug carrier. It has intelligently controlled drug release that can be initiated by pH, ultrasound, magnetism, temperature, irradiation, and electrical effects (Wang et al., 2012b; Wang and Zhao, 2016). Improvement of the site-specific controlled drug-delivery systems was done by combining several responsive systems within one network system through various techniques, such as graft polymerisation, interpenetrating polymer network (IPN), free-radical polymerisation, and the crosslinking of radiation based on the selective and starting material. Given all the stimuli that is responsible for drug delivery, the mixture of pH- and temperature-responsive (pH/T-responsive) has gotten more attention as it can be easily controlled and stimulated for *in-vitro* and *in-vivo* application (Ju et al., 2001; Lo et al., 2005; Mason et al., 2001; Matsusaki and Akashi, 2005; Shi et al., 2006).

Material selectivity for the design of the pH/T-responsive system was one of the most important criteria for the development of a more efficient hydrogel. Recently, hydrogel modification for drug delivery has been focused on hydrogels based on

natural polymer materials especially chitosan. This is because they offer various advantages as compare to synthetic material that promotes neurotoxin and carcinogenic in human body specifically for long circulation in blood system (Saunders et al., 2009). Chitosan, with its excellent properties, was often chosen because it matched the physiological study as well as the synthesis development based on reactive functional group that could be found in the chitosan structure. Chitosan is a pH-sensitive material with a superior compatibility, biodegradable, easily modified with variety shapes, geometries, and formulations (Denkbas and Ottenbrite, 2006; Milosavljević et al., 2011b; Prabaharan and Mano, 2006). The modification on chitosan overcomes fast dissolution in an acidic environment while it positive surface charge not only fasten cellular internalization but also exposes the positive charge to improve the stability of particles (Huang et al., 2017; Sun et al., 2011; Wang et al., 2016b). However, the sensitivity of chitosan hydrogel as a drug carrier is not effective to release drug at accurate time and place. Modification on chitosan hydrogel with other stimuli responsive specifically to pH/T-responsive improved the drug release profile which is close to the responses in a human body and have the best signal in terms of application and safety. Both stimuli also show some changes once the body is attacked by any disease (Yun and Kim, 2012; Zhou and Zhang, 2011).

Several studies were conducted in synthesizing pH/T-responsive hydrogel with chitosan. N-isopropylacrylamide (NIPAM) was a favourable temperature monomer and widely explored in different crosslinking technique to improve the crosslinking network between chitosan and the PNIPAM such as interpenetrating-polymer network (IPN), post-crosslinking technique and crosslink the chitosan by using $CaCl_2$ (Alvarez-Lorenzo et al., 2005; Braga et al., 2019; Chen et al., 2018; Luckanagul et al., 2018). The capability of chitosan with PNIPAM hydrogel network was enhanced by dragged

the VPTT close to the physiological body temperature by using methacrylic acid (MAA). Copolymerization with the MAA monomer widespread the pH range of chitosan which originally more specific to acidic environment to a near basic pH range and at the same time interacted the chitosan from over swelling (Chen et al., 2014b). The crosslinking all the three polymer (chitosan, MAA and NIPAM) proposes a good potential which is able to tolerate a given range of pH to treat tumour cell since the pH is not specific and changing around pH 6.5. Additional on MAA drag the maximum swelling capability from acidic to a higher pH near to the pH tumour cell. The hydrogel's cationic surface charge of chitosan provides advantages in that it improves the cellular internalization and consequently releases drug that could kill the target cancer cell. This served as an alternative to the photodynamic therapy (PDT) in lowering the extent of cell damage during the therapies. Furthermore, in special circumstances such as hyperthermia and neoplasia, this hydrogel was potentially activated so that it could release drug either at a higher temperature (45 ℃ to 50 ℃) or lower pH (pH 5.7 to pH 7.8). It may also work in both situations.

Due to the that good combination between the chitosan, MAA and NIPAM, Kang and Kim (2010) was synthesized chitosan coated with p(MAA-co-NIPAM) in beads form and the stimuli-responsive be more controllable where the drug release become slower. Since the hydrogel is in bead (>10 μm), the particle size limit the capability of the hydrogel to be used for target cancer cell especially for tumour. Positive and negative charge of hydrogel carrier plays an important part where, positive charge carrier enhances the interaction with negatively-charged biological surface and enhances drug uptake while, negative charge carrier decrease cell adsorption of particles (Honary and Zahir, 2013). Therefore, there should continuous development and expansion for a design that presents chitosan with MAA and NIPAM

as an effort to realizing the use of the chitosan-p(MAA-co-NIPAM) hydrogel for drug delivery especially for target cancer cell.

1.2 Problem Statement

Drug delivery can be efficiently controlled by controlling the drug carrier's particle size. The biocompatible features having a particle size within the range of 50 to 500 nm are matching the ability of chitosan-based pH/T-stimuli hydrogel in terms of lengthening the circulation time in the circulation system, improving intracellular target, and increasing cellular uptake (Salatin and Yari Khosroushahi, 2017; Vinogradov et al., 2002). In terms of chitosan-based pH/T-stimuli hydrogel, most reports are concerned with the film and micron beads form (Chen et al., 2014b; Kang and Kim, 2010). Thus, an appropriate method that has a suitable composition must take into consideration in the synthesis process for attaining the desired smaller chitosan-based pH/T-responsive hydrogel.

Involving MAA and NIPAM monomers, it was preferable to utilise the free radical polymerisation method for the preparation of smaller particle sizes hydrogel. The most important problem in the production of the smaller particle size is the aggregation within the particles. Particles that are easily destabilised can be assembled and they may also experience a spontaneous increase the size (Thickett and Gilbert, 2007). Some studies found that the insolubility of the PNIPAM network during polymerization causes the instability on micelle due to the growing polymer chains and the phase separation of the hydrogel particles (Chen et al., 2014a). Despite the fact that the particles showed stability at the early parts of the synthesis, but then started to aggregate after several hours' reaction. The instability property that induces the aggregation may affected the swelling properties, degradation, drug release efficiency,

and drug loading efficiency. An evaluation and investigation to control the polymerization are required to maintain the stability of particle and the function of the overall hydrogel.

Considering the special function of chitosan in hydrogel, it is very important to sustain all the chitosan functions. It is necessarily to increase the chitosan content to ensure the chitosan can be function efficiently. An adequate amount of chitosan prolongs and sustain the compatibility of hydrogel although lots of drugs were released and at the same time some part of chitosan was degraded by enzymes in the body. The content of chitosan also should be sufficient since chitosan acts as a protector to avoid the drug become toxic. Besides, different content of chitosan present in core or shell of hydrogel also affect the stability of the surface charge and swelling/ deswelling of the hydrogel. Meanwhile, there is possibility NIPAM that presence in the hydrogel will continuously shrinkage due to the degradation of chitosan. Hence, different content of chitosan also will affect the drug release profile. Therefore, a suitable method is required to increase the chitosan content of hydrogel and an investigation also needed to ensure the crosslinking of chitosan is vital in maximising its functions.

Another problem of crosslinking the chitosan with MAA and NIPAM is it make the hydrogel become more or less toxic since both of the monomers are synthetic materials (Svirshchevskaya et al., 2016). Therefore, one needs to conduct further investigation to ensure the biocompatibility of the hydrogel.

1.3 Hypothesis of Chitosan-p(MAA-co-NIPAM) Hydrogel

Some varieties are highlighted in synthesis the chitosan-p(MAA-co-NIPAM) hydrogel, especially in terms of size and mechanism of drug release from the hydrogel.

In this study, the synthesis is focused on producing particle size from 500 nm which is an improvement from previous researches that only focus in film and beads. Therefore, first hypothesis in synthesis the chitosan-p(MAA-co-NIPAM) hydrogel is to synthesis the hydrogel by using emulsion polymerization method. The emulsion polymerization method has the potential to produce smaller particle sizes. Monomer composition (MAA, NIPAM and MBA) plays a role in determining the particle formation and growth was considered as the factors affecting particle size. Since there are three monomers involve, design of experiment (DOE) is proposed to get more exact match composition and identifying key factors that need to be controlled to achieve small particles size.

Second hypothesis is about the aggregation of particle during synthesis. The evaluation on the formation of hydrogel at different time intervals is to provide a clear picture of how the hydrogel formed so that the structural changes beyond the reaction time can be observed and the behaviour and properties of the hydrogel can be evaluated. From the evaluation, the synthesis process can be controlled such as the determination of monomer amount, the suitability of monomer time added and to control the formation of the core and shell for a particular monomer.

The last hypothesis in this study is about chitosan control in hydrogel. The mechanism of drug release proposed in this research is through degradation by chitosan and swelling /deswelling of the hydrogel network. In human body, hydrogel will respond and release the drug as changes in pH and temperature. At the first stage of hydrogel in the body, the hydrogel responds from temperature changes either from storage or room temperature and gradually increase to average body temperature (37^{o}C). At the same time, hydrogel respond to the body's pH based on the route

administration used. In this stage, the release of drugs occurs through the swelling/deswelling of the hydrogel.

Prolonged time of hydrogel in the body causes cleavage on chitosan chain. The volume on hydrogel is increasing and allows more drugs to be released. At the same time, shrinkage from NIPAM controls the hydrogel from over swelling and helps in continuation of drug release. In order to achieve such mechanism, it is proposing that chitosan should be located not only at the surface but should also be in the middle of the hydrogel. The advantages of chitosan in the middle will help drug release when the chitosan start to cleavage and also to ensure that the drug is not easily become toxic. Whereas, the presence of chitosan at the surface takes a role to increase it stability and increase cellular uptake during endocytosis by interaction with negative cell membrane.

Therefore, the addition of chitosan through random polymerization allows crosslinking to occur by ensuring chitosan available inside and outside of hydrogel. The amount of chitosan feed also should be considered to maximise the presence of chitosan and the excess amount of chitosan at the end of polymerization will be crosslink as the surface of hydrogel. Furthermore, the chitosan's thickness at the surface may be managed by the chitosan feed that is present at the earlier synthesis. However, this crosslink method influences the physicochemical properties of hydrogel once the reaction time and amount of chitosan feed changed. Therefore, one needs to conduct further investigation to determine the efficacy of random polymer chain on pH/T-responsive behaviour, drug loading efficiency, hydrolysis, biocompatibility of the hydrogel, drug release characteristic and its toxicity.

1.4 Research Objectives

The introduction of pH/T-responsive monomers with chitosan characteristics is presented and the contribution of relevant research supposedly enhances the ability of this hydrogel. Hence, several objectives have been determined for this research work:

1. To synthesise and characterize pH/T-responsive monomers (methacrylic acid (MAA) and N-isopropylacrylamide (NIPAM)) with the presence of chitosan in order to produce particle size below 500 nm.
2. To evaluate the effect of reaction time during the polymerization of chitosan-p(MAA-co-NIPAM) hydrogel on pH and temperature behaviour, hydrolysis, and the efficiency of drug loading and drug release.
3. To investigate the effect of chitosan feed at earlier synthesis of chitosan content on chitosan-p(MAA-co-NIPAM) hydrogel end product, pH and temperature behaviour, hydrolysis, and the efficiency of drug loading and drug release.
4. To determine the mechanism of drug release and its kinetics through chitosan-p(MAA-co-NIPAM) hydrogel.
5. To investigate the toxicity effect of chitosan-p(MAA-co-NIPAM) hydrogel on fibroblast cell line (L929) using AlamarBlue® assay.

1.5 Organisation of the Thesis

This thesis is divided into five chapters:

Chapter 1 describes the general background of hydrogel, pH/T-responsive hydrogel, and pH/T-responsive hydrogel with chitosan. This part also covers the problem statement in the study of pH/T-responsive hydrogel with chitosan, the objectives, and the organisation of the thesis.

Chapter 2 covers the literature related to hydrogel, chitosan, and topics related to the objectives of this thesis including synthesis, hydrogel response, and drug release behaviour.

Chapter 3 describe the raw material specifications, description of synthesis methodology, characterisation, and experimental procedures.

Chapter 4 discusses the screening of particle size analysis, synthesis by surfactant emulsion polymerisation, and also polymerization with different reaction time and chitosan content. The discussion also covers the effect on structure formation, morphology, swelling behaviour, degradation, loading efficiency, and drug release behaviour. Further discussion describes the kinetics and mechanisms of drug release by rifampicin, and the last part is related to the cytotoxicity test on the hydrogel.

Chapter 5 concludes the findings of the thesis based on the objectives stated earlier. Some suggestions are also provided for future study related to the thesis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview

This chapter summarises the introduction of hydrogel and the chronology of hydrogel until chitosan is selected in recent studies for drug delivery application. Some introduction on the preparation and design of chitosan-based hydrogel that contributed to the improvement of the efficiency of recent drug carriers are also deeply reviewed. Recent research related to chitosan-based pH- and temperature-responsive hydrogel studies is also explored in this part.

2.2 Hydrogel

2.2.1 Introduction of Hydrogel

Hydrogel can be defined as swollen materials with crosslinked polymeric network capable of retaining the structure from being dissolved in water (Ahmed, 2015). Hydrogel gets the attention in the biomedical field as hydrogel resembles the cell with hydrophilic functional groups present in several polymers such as $–NH_2$, –OH, –COOH, and $–SO_3H$ (Dwivedi, 2011; Okay, 2009; Satish et al., 2006). Hydrogels are developed and can be classified into three generations based on the important events presented in **Figure 2.1**. The first generation focuses on a simple chemical crosslink in the synthesis of hydrogels with the objectives of increasing swelling properties and producing hydrogels with good mechanical properties. For the second generation, the hydrogels are improved by crosslinking polymers with other monomers for specific function in response to the environment. For the third generation, the hydrogels are developed with more complex crosslinks with the aim of producing

'smart' multi-responsive hydrogels to the environment (Buwalda et al., 2014; Yahia et al., 2015). A smart hydrogel is defined by activating a polymer network with either physical stimuli such as temperature, electric field, solvent composition, light, pressure, and sound and magnetic fields, or chemical stimuli including pH, ion, and specific molecular recognition events such as glucose, enzyme and antigens (Ebara et al., 2014; Parodi et al., 2016).

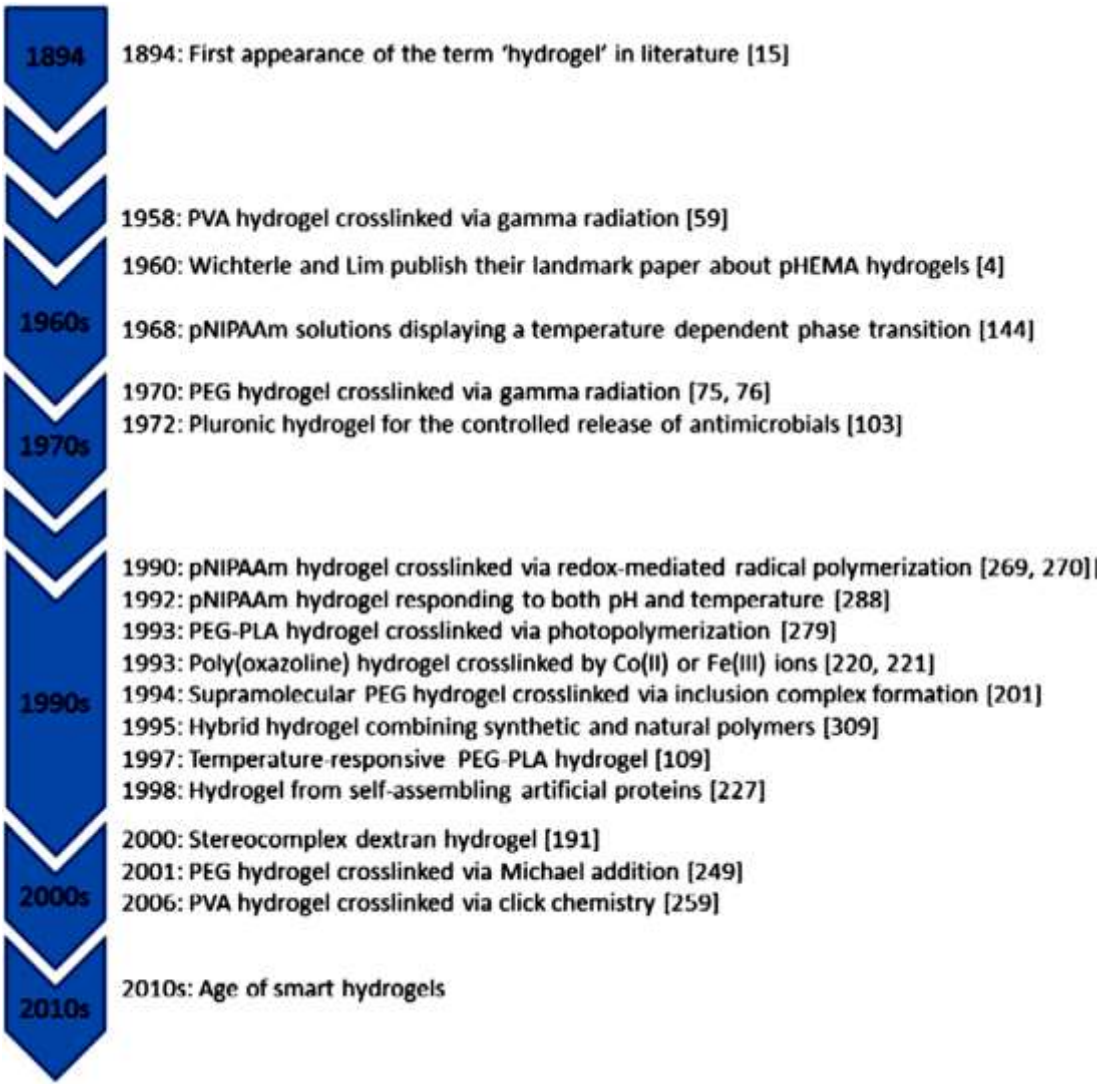

Figure 2.1: Timeline presenting the most important events in the history of hydrogel research (Buwalda et al., 2014).

Prior to the utilisation of smart hydrogel in drug delivery, temperature and pH stimuli are predominantly studied among researchers, where temperature stimuli are capable of collapsing and removing solvents while pH stimuli expand hydrogels by absorbing solvents (Soppimath et al., 2002). The single stimuli-responsive polymer, either from natural or synthetic polymer, does not fix the pathological condition and it requires another stimulus to control drug release. The combination of both stimuli allows the activation of hydrogel at specific temperature and pH efficiently and thus giving the hydrogel's name as a smart material. Although hydrogel is known as a biocompatible material based on its hydrophilic properties, this multi-responsive hydrogel still induces toxicity and could be dangerous if it stays longer in the human body. In some cases, especially for site-targeting drug delivery, biocompatibility is very critical since the drug is carried longer in the blood circulation system before it is safely delivered. The combination of a hydrophobic polymer hydrogel such as N-isopropylacrylamide (NIPAM) makes the hydrogel as a foreign substance (Wang et al., 2012a). Thus, this increases toxicity and reduces the bioavailability of the drug (Naahidi et al., 2013).

An initiative work was done by combining a hydrophilic natural polymer and a synthetic polymer because the hydrophilic natural polymer possesses a higher degree of biocompatibility (Cho et al., 2015). A natural polymer that comprised of cationic and anionic polymers and present as a pH-sensitive material was crosslinked with temperature-sensitive materials. Recently, natural polysaccharide-based materials such as chitosan (Bai et al., 2018), cellulose (Choe et al., 2018), dextran (Chandel et al., 2018; Prusty and Swain, 2018), alginate (Cong et al., 2018), and hyaluronic acid (Colter et al., 2018) have attracted more attention in drug delivery using hydrogels with several different objectives. The crosslink between both of the polymer in the

formation of a smart hydrogel improves the properties and biocompatibility of a hydrogel.

2.2.2 Hydrogel in Drug Delivery

Drug delivery can be classified into passive and active targeting. Passive targeting accumulates and delivers a drug by the natural response of enhanced permeability and retention (EPR) while in active targeting, a drug is carried and delivered to the target cell by the functional group of the carrier with ligand and binds with the receptor. A good functionalisation of hydrogel is advantageous to control drug release in the context of active targeting (Mishra et al., 2016). An attractive structure and properties of hydrogel with good colloidal stability, simple preparation, easy functionalisation, and protection of the drug from adverse environment give rise to the use of hydrogel in drug delivery studies (Ebara et al., 2014; Saunders et al., 2009).

Stimuli-responsive hydrogel that mimics normal physiological hydrogel allows a certain amount of drug release based on the physiological requirement. Several forces activate the hydrogel to response in different ways such as solubilisation/precipitation, degradation, phase transition, micellisation, and swelling/collapsing (Aguilar et al., 2007; De las Heras Alarcón et al., 2005; Kumar et al., 2007). All the responses are exactly related to the chemical (crosslinking) and physical (porosity) forms of hydrogel by different external and internal stimuli, which are also related to the release rate of drug (Srivastava et al., 2015). **Figure 2.2** listed different ways of drug being delivered through hydrogels.

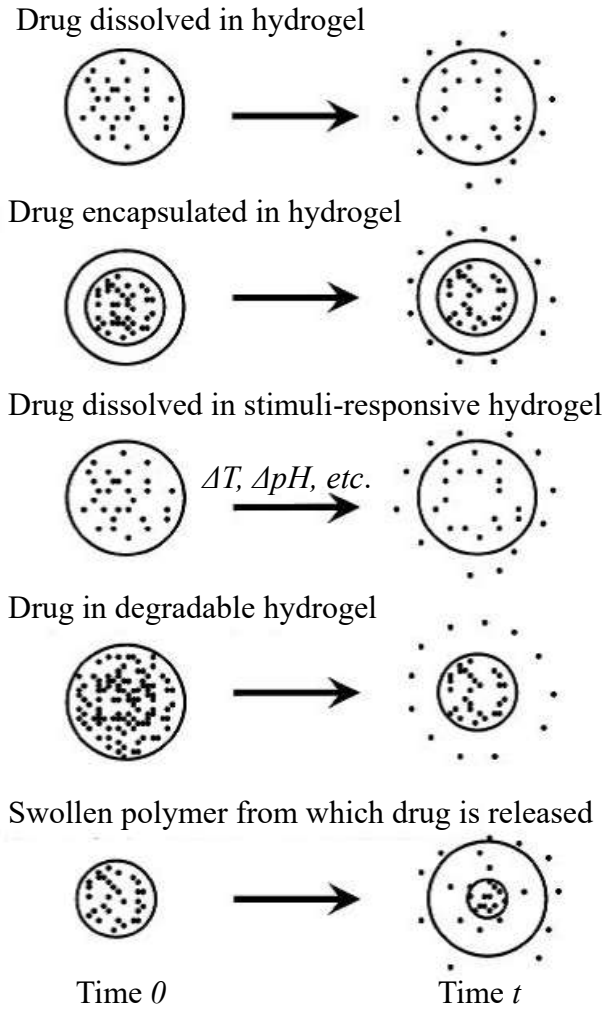

Figure 2.2: Drug delivery mechanism through hydrogel (Peter et al., 2012).

2.2.2(a) Temperature-responsive Hydrogel

A temperature-responsive polymer exhibits a volume-phase transition as a response at a critical solution temperature according to their composition. By considering the volume phase transition temperature (VPTT) that is most suited with hydrogel, the polymer undergoes either swelling or collapsing upon heating. For a thermophilic hydrogel such as poly(N-acryloylglycinamide), the volume of hydrogel increases and it makes the hydrogel swells. Meanwhile, thermophobic hydrogels such as polyethylene glycol (PEG) and poly(N-isopropylacrylamide) (PNIPAM) reduce the volume and collapse the hydrogels (Li et al., 2013). At a lower VPTT, a thermophobic

hydrogel is soluble in a solvent and it is the main attribute that is mostly used for a temperature-responsive hydrogel in drug delivery application. Hydrogel potentially reduces non-specific protein binding, improves biocompatibility, prevents coagulation/aggregation, and enables sensing capabilities or modulating solubility (Gibson and O'Reilly, 2013). Instead of other temperature-responsive polymers such as poly(N,N-diethylacrylamide), poly(N-vinylalkylamide), poly(N-vinylcaprolactam), phosphazene derivatives, pluronics, and tetronics (Priya James et al., 2014), PNIPAM shows different concepts and makes it the first priority among temperature-responsive polymers.

PNIPAM hydrogels have been extensively studied in the context of drug delivery since the phase transition occurs near the human body temperature at approximately 32 °C. The phase transition also shows a wide range of phase transition temperatures, as well as a fast, tunable, and sharp change in hydrophilicity/hydrophobicity, and this approaches leads to fast drug delivery to the target (De las Heras Alarcón et al., 2005; Yoshida et al., 1995). The most advantageous feature of PNIPAM is the capability to adjust the phase-transition temperature higher than the body temperature, which is suitable for tumour, fever, and hyperthermia cases (Lue et al., 2011). This temperature-responsive polymer balances the hydrophilic-hydrophobic structure and the chain collapses. This concept was first introduced as a surface of a cell substrate, which makes it easy to switch by detaching and keeping the cell intact in the enzymatic treatment procedure (Yamada et al., 1990). The temperature-responsive polymer was studied for injection route delivery, where the hydrogel was soluble and could be easily injected into the human body by forming a gel once the hydrogel reached the body temperature (Jeong and Gutowska, 2002). The polymer was also alternatively applied for switching and collapsing the polymer

to expose the ligand in binding with the receptor for cell internalization (Mastrotto et al., 2011). In some case study especially for nanohydrogel, a temperature-responsive polymer with a crosslinked copolymer network containing a hydrophilic drug was collapsed and the release of the drug was controlled (Chung et al., 1999). The criteria for consideration in designing a small particle size of temperature-responsive hydrogel generally apply this concept for drug delivery.

2.2.2(b) pH-responsive Hydrogel

Generally, pH-responsive hydrogel has the ability to respond to the physiological body environment, which leads to the change in the charge of hydrogel chain, and tends to swell the network and releases the drug. Siegel (2014) mentioned three factors related to hydrogel swelling: polymer elasticity, polymer/solvent interaction, and ion osmotic pressure. Meanwhile, the swelling ability especially for an ionic hydrogel can be classified into two factors: (i) hydrogel structural properties such as concentration, pK_a or pK_b of the ionisable group, degree of ionisation, crosslink density, and the hydrophilicity or hydrophobicity of the pH-responsive polymer, and (ii) properties of swelling medium such as counter ion, pH, and ionic strength (Mahdavinia et al., 2004).

Different pendant groups of hydrogel, either acidic or basic pendant groups, make the hydrogel to swell upon contact with the swelling medium. Usually, acidic and basic pendant groups consist of –COOH (carboxyl group) and $–NH_2$ (amino group), respectively. Both groups are ionised at physiological pH and other body compartments since each compartment in a body has a different pH level. At lower pH medium, the amino group is protonated ($–NH_3^+$) while at higher pH medium, the carboxylic acid group is ionised ($–COO^-$). From ionised to unionised or opposite with control by hydrogel composition and crosslink density, the resultant behaviour is a

response that causes hydrogel to swell and deswell. The entrapped drug is then released either by diffusion or chemically-controlled mechanism with the display release rates are pH dependent (Bencherif et al., 2009). The mechanism of hydrogel ionisation in the release of drug is simplified as shown in **Figure 2.3**

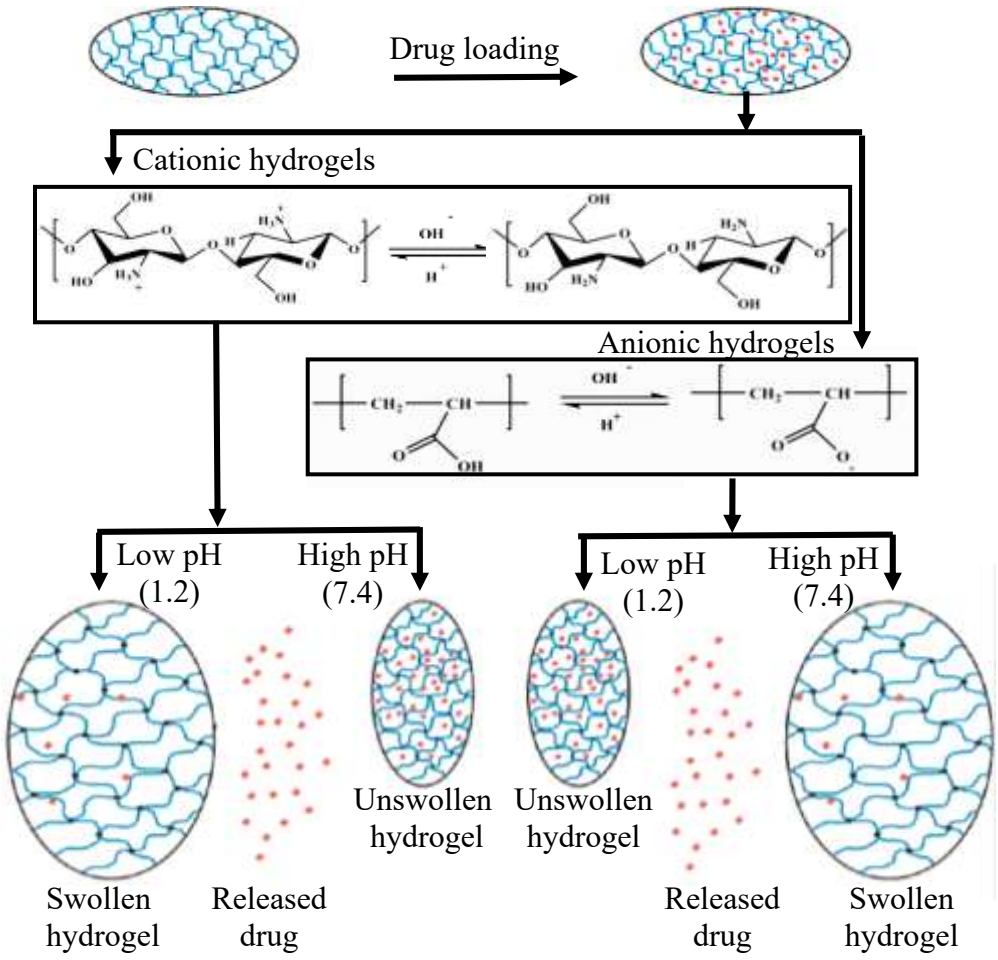

Figure 2.3: pH dependent ionization acidic and basic pendant group and drug release mechanism (Rizwan et al., 2017).

pH-responsive polymer hydrogels are derived from natural and synthetic materials. Chitosan, guar gam, carrageenan, dextran, xanthan, cellulose, and alginate are among the natural polymers used to produce pH-responsive polymers whereas among the synthetic polymers used include poly(acrylamide) (PAAm), poly(acrylic acid) (PAA),

poly(methacrylic acid) (PMAA), and poly(dimethylaminoethyl methacrylate) (PDMAEMA) (Bajpai et al., 2008; Gupta et al., 2002; Rizwan et al., 2017). Among the polymers, PMAA is well known as a sharp, dramatic, and fast kinetics swelling polymer compared to PAA (Brannon-Peppas and Peppas, 2006) (Brazel and Peppas, 1996). PMAA also has a good capability in controlling solute permeation influenced by the environment medium (Brazel and Peppas, 1996). In drug delivery, the increase of MAA composition in copolymerisation synthesis potentially enhances the hydrolysis in intestinal fluids (SIF, pH 7.0) by the ionisation of –COOH group and enhances the rate of drug release (Bartil et al., 2007). Generally, PMAA is crosslinked with other stimuli polymers or with another pH-sensitive polymer to improve the function of PMAA in controlled drug release.

2.3 Chitosan-based Hydrogel

2.3.1 Introduction of Chitosan

Chitosan is one of the natural polysaccharides that received special attention due to its abundance, low production cost and high demand in manufacturing industries (He et al., 2016; Peter, 1995). Chitosan is obtained from chitin of different sources, such as sea animals, insects, and microorganisms (Mathur and Narang, 1990). Chitin consists of N-acetyl-D-glucosamine and D-glucosamine units with one amino and two hydroxyl groups (Agrawal et al., 2010). The protonation of amino group below pKa from NH_2 to NH_3^+ allows chitosan to be soluble in an acidic solution, which can be achieved by dissolving chitosan in weak acids such as hydrochloric acid, lactic acid, and acetic acid as represented in **Equation 2.1** (Rinaudo et al., 1999).

$$Cs\text{-}NH_2 + H_3O^+ \rightarrow Cs\text{-}NH_3^+ + H_2O \quad (2.1)$$

This special feature makes chitosan the only cationic natural polymer that has the ability to form polyelectrolyte complexes once it is associated to simple carboxylic acids such as formate, acetate, lactate, malate, citrate, glyoxylate, pyruvate, glycolate, and ascorbate (Muzzarelli and Muzzarelli, 2005). The cationic nature of chitosan makes it versatile and flexible for modification (Alvarez-Lorenzo et al., 2013; Rinaudo et al., 1999; Singla and Chawla, 2001). The solubility of chitosan with an active functional amino group enables chitosan to be used in various applications such as in solutions, gels, films, and fibres (Rinaudo, 2006).

2.3.2 Introduction of Chitosan Hydrogel for Drug Delivery

The selection of chitosan for drug delivery receives attention because chitosan has biological properties such as biocompatible, biodegradable to normal body constituents, safe and non-toxic, bioabsorbable, haemostatic, bacteriostatic, fungistatic, spermicidal, anticancerogen, anticholesteremic, antiulcer, and antiacid (Abdelhalim, 2006). The main characteristic of chitosan is its cationic nature. The special positive charge attributes to mucoadhesive property, biocompatibility, and many advantageous characteristics that are promising when chitosan is preferred for drug delivery application. The mucoadhesion of chitosan can prolong the time of loaded drug being released from the system and provide localised drug delivery. The adhesion is applicable for mucosal drug delivery, especially with mucosal glycoproteins that are negatively charged. The positive charge of chitosan enhances the interaction with other negatively-charged biological surfaces (macromolecules, nucleic acids, and proteins) and thus enhances drug uptake as shown in **Figure 2.4**

(Miao et al., 2018; Peppas and Sahlin, 1996). It also has the ability to bind with the tissues suitable in specific drug delivery (Lueßen et al., 1996; Zhao et al., 2014).

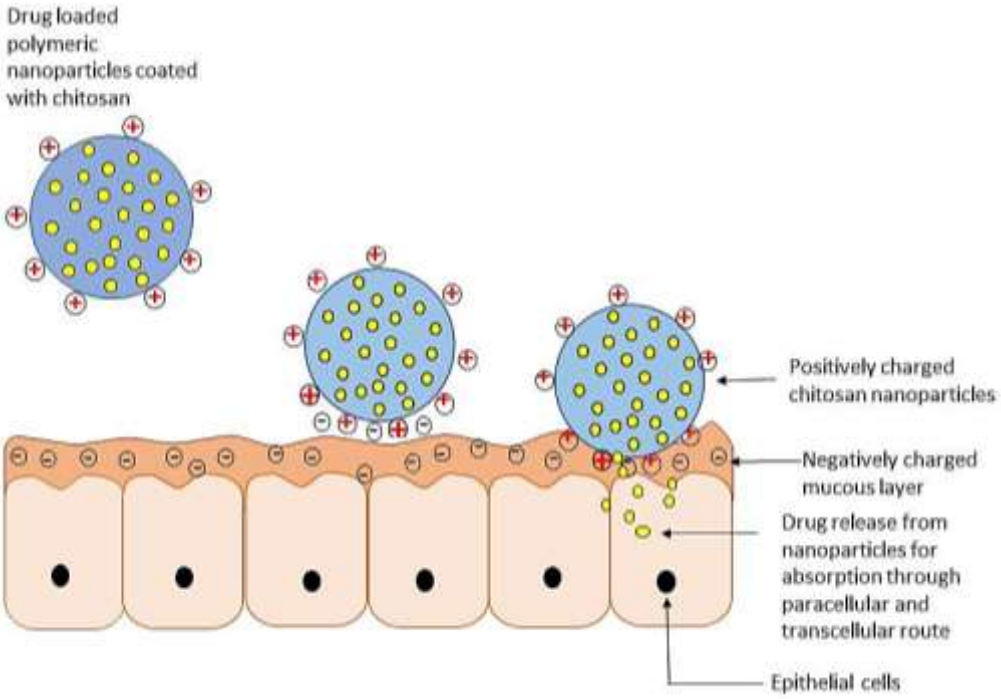

Figure 2.4: Schematic represent chitosan with positive charge interacted with negative charge biological surface enhances drug uptake (Mohammed et al., 2017)

All the characteristics are more beneficial once chitosan is modified in hydrogel form. Easy modification by the presence of functional groups in chitosan produces a polymer network with high swelling ability that is capable of absorbing a large amount of water (Rani et al., 2010). The hydrophilicity of chitosan hydrogel is enhanced as chitosan is a pH-sensitive material with a superior compatibility and also biodegradable (Milosavljević et al., 2011b; Prabaharan and Mano, 2006). Chitosan hydrogels have been prepared with a variety of different shapes, geometries, and formulations that include liquid gels, powders, beads, films, tablets, capsules, microspheres, microparticles, sponges, nanofibrils, textile fibres, and inorganic composites (Denkbas and Ottenbrite, 2006). The modification on chitosan overcomes

fast dissolution in an acidic environment, especially in stomach and increases the percentage of drug loaded for delivery (Sun et al., 2011). Easy modification of chitosan also gives more benefits in controlling hydrogel synthesis by designing core-shell structure. Positive surface charge of a shell affects the erosion in exposing negative surface charge of nanoparticles in reaching tumour sites (Huang et al., 2017). Positive surface charge not only fasten cellular internalization but also exposes the positive charge to improve the stability of particles, especially for smaller particle size of hydrogel (Wang et al., 2016b). The main reason for selecting chitosan is its biocompatibility properties. Chemical agents, as well as synthetic and hydrophobic polymers, can potentially produce toxicity in the system. Therefore, biocompatible chitosan crosslink is preferred for further applications (Pamfil and Vasile, 2018). The modification of chitosan can be applied by crosslinking chitosan with either chemical or physical crosslink. Crosslinking with stimuli-responsive polymer hydrogel can make chitosan as a smart hydrogel for controlled drug delivery.

2.3.3 Stimuli Responsive Chitosan-based Hydrogel

Based on previous studies of chitosan-based hydrogel drug delivery, pH-sensitive polymer is the most interesting stimulus, and chitosan is a pH-sensitive material. Temperature-, glucose-, electric-, light-, and magnetic-responsive materials have been studied by researchers by crosslinking chitosan with the specific stimuli. Among the stimuli, pH and temperature are the most effective stimuli that are close to the responses in a human body and have the best signal in terms of application and safety. Both stimuli also show some changes once the body is attacked by any disease (Yun and Kim, 2012; Zhou and Zhang, 2011).

2.3.3(a) Chitosan-PMAA hydrogel for pH-responsive Drug Delivery

Chitosan-based hydrogel is commonly used as a pH-sensitive material for drug release application in acidic environment. Chitosan is crosslinked either with another pH-sensitive polymer in basic environment or other polymers designed for multi-sensitive polymer hydrogel. A study of chitosan crosslink with MAA monomer is of much interest as the production of chitosan and MAA was firstly reported in microsphere and microparticle forms. However, the sample preparation was almost similar with chitosan and MAA for hydrogel form. Basically, chitosan was dissolved in either acetic acid solution or methacrylic acid solution and free radical was added for polymerisation (Bashir et al., 2016; de Moura et al., 2008; Heidari et al., 2013). In contrast to hydrogel form, a crosslinker was added, such as N,N′-methylenebisacrylamide (MBA) to form a three-dimensional network. Chitosan crosslinked with MAA showed higher ionisation at pH 2.30, and as pH increased, the degree of swelling decreased due to the increase in crosslinking density by the interaction of $–NH_3^+$ with $–COO^-$. From pH 4.5 to pH 6.8, maximum ionisation of chitosan was achieved and a compact structure was produced with very small changes in volume. The mechanism of swelling is shown in **Figure 2.5**.

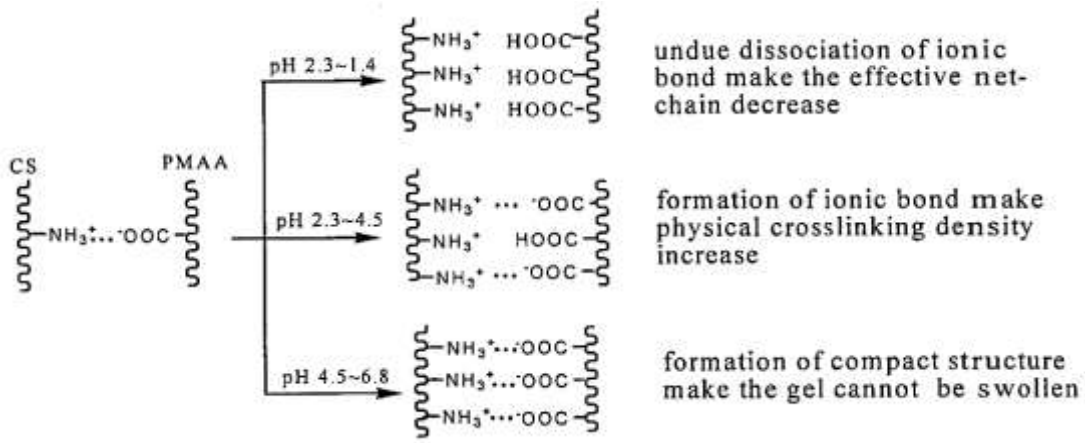

Figure 2.5: Structure change of polyelectrolyte complex in various pH solutions (Chen et al., 2005).

Chitosan crosslinked with PMAA hydrogel shows intrinsic biological properties that pass the cytotoxicity test for the viability of L929 cells (Aycan and Alemdar, 2017; Lehr et al., 1992; Qu et al., 2017). The hydrogel has the capability of ionic strength response since it changes drastically due to the osmotic pressure of the ambient solution (Feil et al., 1993; Khan et al., 2013a; Milosavljević et al., 2011a; Zhang and Peppas, 2000). The response of MAA to the swelling behaviour was rapid at appropriate pH although a small amount of MAA ratio was used.

2.3.3(b) Chitosan-PNIPAM hydrogel for Temperature-responsive Drug Delivery

An excellent characteristic of NIPAM, which is its phase transition near to the human body temperature at approximately 32 °C, has attracted researchers to continue their study to improve the primary weakness of NIPAM. PNIPAM is one of the hydrophobic polymers with the potential to absorb unwanted protein in a human body (Boutris et al., 1997; Kawaguchi et al., 1992; Nichols et al., 2009). It is also non-biodegradable and thus promotes neurotoxin and produces carcinogenic or teratogenic toxicity (Fang et al., 2008; Saunders et al., 2009). NIPAM with its comonomer also has poor mechanical properties and is less compatible with organs and tissues (Zhao et al., 2011). As an alternative to this problem, natural polymer especially chitosan has been selected and incorporated with NIPAM either by grafting or interpenetrating network (Li et al., 2012; Milichovsky, 2010).

The copolymerisation of chitosan and PNIPAM is an excellent idea for producing a smart hydrogel because the temperature-responsive PNIPAM is capable of improving chitosan hydrogel, which is only responsive to pH change. The smart hydrogel also sustains the temperature sensitivity of PNIPAM while improving its biocompatibility and biomedical properties (Fernández-Gutiérrez et al., 2016). The

research done by Wang et al. (2000) found that copolymerised chitosan-PNIPAM formed thermally-reversible hydrogel and exhibited a lower critical solution temperature (LCST) at 32–35 °C in aqueous solutions. The crosslink between chitosan and PNIPAM shows that deswelling/shrinkage occurred in the temperature range of 36–39 °C (Khan et al., 2015). This proves that the LCST of PNIPAM can be adjusted from 31–32.8 °C to around the physiological temperature of 37 °C. However, LCST is also affected by the ratio of hydrophilic and hydrophobic monomers in thermosensitive polymers (Salehi et al., 2009).

2.3.3(c) Chitosan-based Hydrogel with MAA and NIPAM monomer for Drug Delivery

Copolymerisation of dual stimuli-responsive polymers, especially pH- and temperature-responsive (pH/T-responsive) polymers, is commonly applied for drug carrier synthesis to achieve very precise, controlled drug delivery. Although chitosan-PMAA and chitosan-PNIPAM hydrogels show drug carrier characteristics with controlled drug release, there are still some limitations in the system. The combination of PNIPAM with chitosan in the form of interpenetrating polymer network (IPN) has faster drug release especially at lower pH. There is also no crosslink between chitosan and PNIPAM, which makes the chitosan to abandon the network especially in washing procedure. Several works have been done to improve the limitation by crosslinking chitosan with a crosslink agent in a post-crosslinking technique (Alvarez-Lorenzo et al., 2005) and crosslinking chitosan with $CaCl_2$ (Chen et al., 2014b). Although the ionisation of chitosan does not disturb the VPTT of NIPAM, the hydrogel has a greater affinity with the anionic drug based on the ionic interaction with the amino group of chitosan.