รายงานวิจัยฉบับสมบูรณ์

ไฮโดรเจล “ฉลาด” ชนิดคาร์บอกซีเมทิลไคโตซานกร๊าฟด้วยพอลิอะคริลิกแอซิดและพอลิเอ็นไอโซโพรพิลอะคริลาไมด์

“Smart” hydrogel based on carboxymethylchitosan grafted with poly(acrylic acid) and poly(*N*-isopropylacrylamide)

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สัญญาเลขที่ R2557B065

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"Smart" hydrogel based on carboxymethylchitosan grafted with poly(acrylic acid) and poly(*N*-isopropylacrylamide)

Abstract

Carboxymethylchitosan (CMC) hydrogel containing thermo-responsive poly(*N*-isopropylacrylamide) (poly(NIPAAm)) and pH-responsive poly(acrylic acid) (poly(AA)) was prepared *via* a free radical polymerization in the presence of hexamethylene-1,6-di-(aminocarboxysulfonate) crosslinker. A proper ratio of CMC to NIPAAm and AA used in the reaction was investigated such that the thermo- and pH-responsive properties of the hydrogel were retained. At the temperature lower than the lower critical solution temperature (LCST) of poly(NIPAAm) (10°C, LCST of poly(NIPAAm) =32°C), the high equilibrium water content of the hydrogels was observed indicating the swollen state of the hydrogel. On the other hand, the hydrogel deswelled at the temperature above its LCST (50°C). pH-responsive behavior of the hydrogels was also investigated. The hydrogel deswelled in the solution having pH 4 and swelled in those having pH 10. This was attributed to the formation of $-COO^-$ of poly(AA) in the hydrogel structure in the basic solution. Water swelling properties of the hydrogel and the releasing rate of an entrapped drug were highly dependent on temperature and pH of the solutions. The hydrogels were not toxic and showed antibacterial activity against *Straphylococcus aureus* (*S. aureus*). The pH- and thermo-responsive properties of this novel "smart" hydrogel might be efficiently used as dual triggering mechanisms in drug controlled-release applications.

Keyword: carboxymethylchitosan; hydrogel; poly(*N*-isopropylacrylamide); poly(acrylic acid); thermo-responsive; pH-responsive

ไฮโดรเจล “ฉลาด” ชนิดคาร์บอกซีเมทิลไคโตซานกร๊าฟด้วยพอลิอะคริลิกแอซิดและพอลิเอ็นไอโซโพรพิลอะคริลาไมด์

บทคัดย่อ

งานวิจัยนี้ศึกษาการเตรียมไฮโดรเจลชนิดคาร์บอกซีเมทิลไคโตซาน (CMC) ที่มีส่วนประกอบของพอลิเอ็นไอโซโพรพิลอะคริลาไมด์ (poly(NIPAAm)) ซึ่งสามาตรตอบสนองต่อการเปลี่ยนแปลงอุณหภูมิและพอลิอะคริลิกแอซิด (poly(AA)) ที่สามารถตอบสนองต่อการเปลี่ยนแปลงสภาวะความเป็นกรดเบส (pH) ได้ โดยในขั้นตอนการสังเคราะห์จะใช้ปฏิกิริยาอนุมูลอิสระและใช้เฮกสะเมทิลีนไดอะมิโนคาร์บอกซี่ซัลโฟเนตเป็นสารเชื่อมโยงตาข่าย การปรับอัตราส่วนโดยโมลระหว่าง CMC, NIPAAm และ AA ซึ่งเป็นมอนอเมอร์จะทำให้ได้ไฮโดรเจลที่ตอบสนองต่อการเปลี่ยนแปลงอุณหภูมิและสภาวะความเป็นกรดเบสของสารละลายได้ จากผลการทดลองพบว่าไฮโดรเจลมีค่าเปอร์เซ็นต์การบวมน้ำที่สูงมากเมื่ออุณหภูมิของสารละลายต่ำกว่าค่าอุณหภูมิสารละลายวิกฤติ (LCST) ของพอลิเอ็นไอโซโพรพิลอะคริลาไมด์ (32°C) ซึ่งแสดงถึงการที่ไฮโดรเจลอยู่ในสภาวะที่บวมน้ำ (swollen state) และจะมีค่าการบวมน้ำที่ต่ำเมื่ออุณหภูมิต่ำกว่าค่าอุณหภูมิสารละลายวิกฤติ (50°C) นอกจากนี้จากผลการทดสอบสมบัติการตอบสนองต่อการเปลี่ยนแปลงสภาวะความเป็นกรดเบสของน้ำพบว่า ไฮโดรเจลจะหดตัวที่ pH 4 และจะบวมตัวที่ pH 10 ทั้งนี้เนื่องจากการเกิดเป็นคาร์บอกซิลเลต (COO^-) ในสายโซ่ของพอลิอะคริลิกแอซิดในสภาวะที่สารละลายเป็นเบส ดังนั้นจะพบว่าสมบัติการบวมน้ำและอัตราการปลดปล่อยยาที่ถูกตรึงในไฮโดรเจลจะขึ้นอยู่กับอุณหภูมิและสภาวะความเป็นกรดเบสของสารละลาย แผ่นไฮโดรเจลชนิดใหม่นี้ไม่มีความเป็นพิษและสามารถแสดงสมบัติการต้านการเจริญเติบโตของเชื้อแบคทีเรียชนิด *Straphylococcus aureus* (*S. aureus*) ได้ ดังนั้นแผ่นไฮโดรเจลฉลาดนี้สามารถนำไปใช้ในการประยุกต์ใช้ด้านการควบคุมการปลดปล่อยยาโดยอาศัยสมบัติการตอบสนองต่อการเปลี่ยนแปลงอุณหภูมิและสภาวะความเป็นกรดเบสของสารละลายได้

คำสำคัญ คาร์บอกซิลเมทิลไคโตแซน ไฮโดรเจล พอลิเอ็นไอโซโพรพิลอะคริลาไมด์ อะคริลิกแอซิด การตอบสนองต่อการเปลี่ยนแปลงอุณหภูมิ การตอบสนองต่อการเปลี่ยนแปลงสภาวะความเป็นกรดเบส

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Executive summary

บทสรุปผู้บริหาร

This work presents the preparation of carboxymethylchitosan (CMC) hydrogels containing thermo-responsive poly(*N*-isopropylacrylamide) (poly(NIPAAm)) and pH-responsive poly(acrylic acid) (poly(AA)) *via* a free radical polymerization in the presence of hexamethylene-1,6-di-(aminocarboxysulfonate) crosslinking agents. A proper ratio of CMC to NIPAAm and AA used in the reaction was investigated such that the thermo- and pH-responsive properties of the hydrogels were obtained. Water swelling of the hydrogels was improved when the solution pH was in basic conditions (pH 10) or the temperature was below its lower critical solution temperature (LCST). Effects of the change in solution temperature and pH on water swelling properties of the hydrogel as well as the releasing rate of an entrapped drug were also investigated. The hydrogels were not toxic and showed antibacterial activity against *Straphylococcus aureus* (*S. aureus*).

Therefore, we herein deconstrtes a simple strategy to prepare "smart" hydrogels based on CMC. These hydrogels containing thermo-responsive poly(NIPAAm) and pH-responsive poly(AA) were synthesized *via* a simple, cost effective and environmentally friendly free radical polymerization in water. The hydrogels showed responses after exposure to temperature and pH stimuli due to the change in their structures. Due to their non-toxic properties, these novel "smart" hydrogels might be potentially applicable in biomedical uses, such as the hydrogels having dual triggering release mechanisms for controlled drug release applications. In addition, these hydrogels might also be beneficial in the applications requiring antibacterial properties.

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CHAPTER I

INTRODUCTION

Rationale for the study

Recently, hydrogel has been widely used in many potential applications such as medicine [1], biotechnology [2], industry [3] and environmental science [4]. Hydrogel is a network of hydrophilic polymers that can swell and absorb large amount of aqueous solution while maintaining its structure [5]. Three-dimensional network of hydrogel can be formed by crosslinking polymer chains through either covalent bonding, hydrogen bonding, van der Walls interaction or physical entanglements [6-8]. Interestingly, hydrogel can also control drug releasing behavior by changing the gel structure in responses to the change in its environment. The hydrogel containing such "sensor" properties can undergo reversible volume phase transition upon only a few minute changes in the environmental condition. This environment-responsive hydrogel is also called "smart" or "intelligent" hydrogel [9-11].

During these recent years, carboxymethylchitosan (CMC), a natural amphoteric polyelectrolyte [12], has attracted considerable interest in a wide range of biomedical applications, such as wound dressings, artificial bone and skin, and bacteriostatic agents due to its good biocompatibility and low toxicity [13, 14]. CMC is a chitosan derivative having carboxymethyl substituents on some of amino and/or primary hydroxyl sites of the glucosamine units of chitosan. It shows good pH and ion sensitivity in aqueous solutions due to abundant ionizable -COOH and $-NH_2$ functional groups in its structure. CMC is well soluble in water [15, 16], while CMC hydrogel (a crosslinked form) swells significantly in basic solutions and shrinks in acidic solutions. Therefore, CMC hydrogel has been widely studied for use in controllable drug delivery.

In addition, thermo-responsive polymers have been increasingly investigated for use in several biomedical applications [17] such as selective bio-separation [18, 19], smart bioactive surfaces [20, 21] and phase separation immune-assays [22, 23]. These polymers usually consist of a lower critical solution temperature (LCST), which is the characteristic phase transition temperature of each polymer. Below the LCST,

the enthalpy term relating to the hydrogen bonding between polar groups of the polymer and water molecules dominates, leading to dissolution of the polymer in water. Above the LCST, the entropy term relating to hydrophobic interactions among the polymers dominates [24], resulting in precipitation of the polymer in water. Poly(*N*-isopropylacrylamide) (poly(NIPAAm)) is the best-known thermo-responsive polymer in this class because it exhibits the LCST at 32°C [25, 26], which is somewhat close to that of the human body (37°C) [27-29], in aqueous solution. Therefore, Poly(NIPAAm), a thermo-responsive polymer [30], was used widely in the field of chemistry, material and biotechnology [31].

Another class of "smart" polymer that is now of great interest is the polymer having pH-responsive properties. These pH-responsive polymers usually are polyelectrolytes [32, 33] that bear weak acidic or basic groups in their structure [34], allowing them to either protonate or deprotonate in response to the change in their environmental pH. The typical example of this class of polymers is poly(carboxylic acid) in particular poly(acrylic acid) (poly(AA)). It swells in basic pH solutions due to the formation of carboxylate anions and collapses in acidic pH solutions [35, 36] because the carboxylic groups are protonated and unionized.

Scope of the study

The aim of this work was to develop a novel thermo- and pH-responsive hydrogel based on CMC with dual triggering mechanisms for drug controlled release. This hydrogel composed of biocompatible CMC, thermo-responsive poly(NIPAAm) and pH-responsive poly(AA) using a water soluble crosslinking agent (hexamethylene-1,6-di-(aminocarboxysulfonate) or HDA) to form a semi-interpenetrating polymer network (semi-IPN) (Figure 1). Water contact angle measurement was carried out to study surface properties of the hydrogel and scanning electron microscopy (SEM) was performed to investigate its morphology. Swelling characteristics of the hydrogel as a function of solution temperature and pH were investigated. The release profiles of indomethacin, a model drug, from the hydrogels were studied. Additionally, cytotoxicity and antibacterial activity of the hydrogel were also investigated.

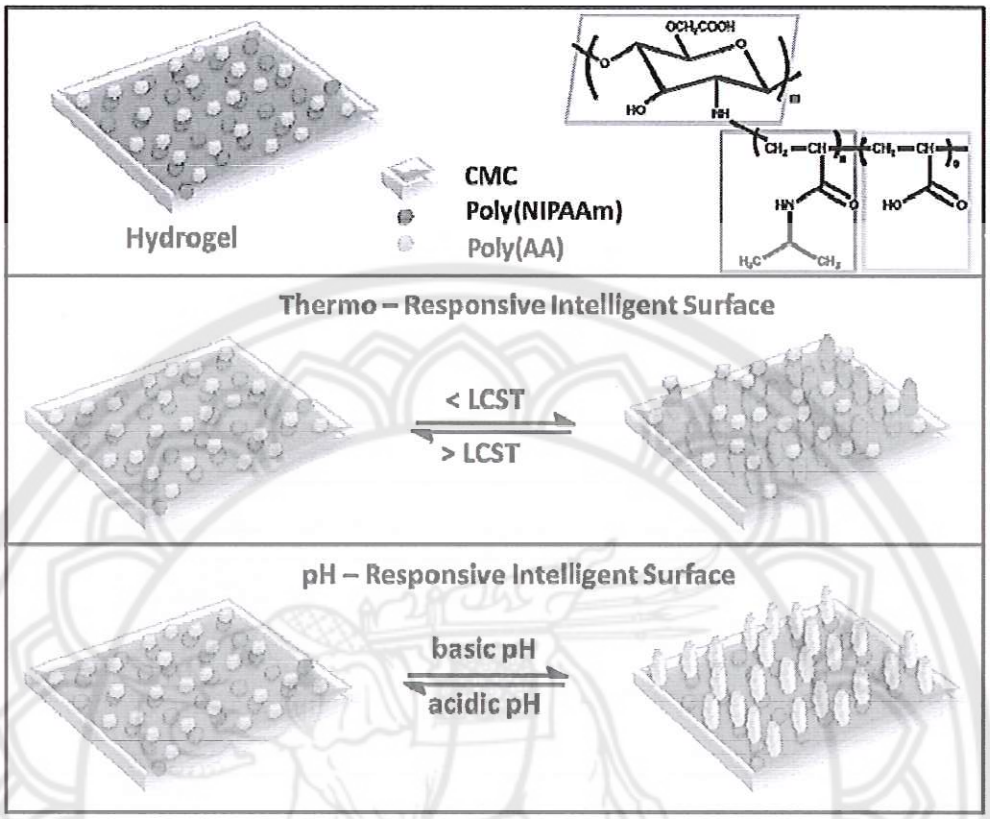


Figure 1 (A) Modification of CMC hydrogel with thermo-responsive poly(NIPAAm) and pH-responsive poly(AA), (B) and (C) ideal swelling/deswelling behavior upon changing its environmental temperature and pH, respectively

CHAPTER II

LITERATURE REVIEWS

Introduction to hydrogel

Hydrogels have been extensively studied in the academic and industrial for development of the smart drug delivery systems [7]. A hydrogel is a kind of hydrophilic polymers network system that can hold a large amount of water in the interspaces of network while maintaining its structure [6, 37]. The hydrogels can undergo reversible volume phase transition and response to external stimuli such as solvent [38], pH [39], temperature [40], ionic concentration [41], electric field [42] and light irradiation [43]. They have been widely investigated in the volume phase transition in different hydrogel systems, especially poly(*N*-isopropylacrylamide) (poly(NIPAAm)). They response to different environmental stimuli and thus have potential application in controlled drug delivery. This type of hydrogel with environment-sensitive properties is also called "Intelligent" or "Smart" hydrogel. It has been demonstrated that the volume phase transition of hydrogel is the result of the sole or combination of four kinds of molecular interactions; ionic interaction, hydrophobic interaction, hydrogen bonding and van der Waals force [44].

Synthetic hydrogel

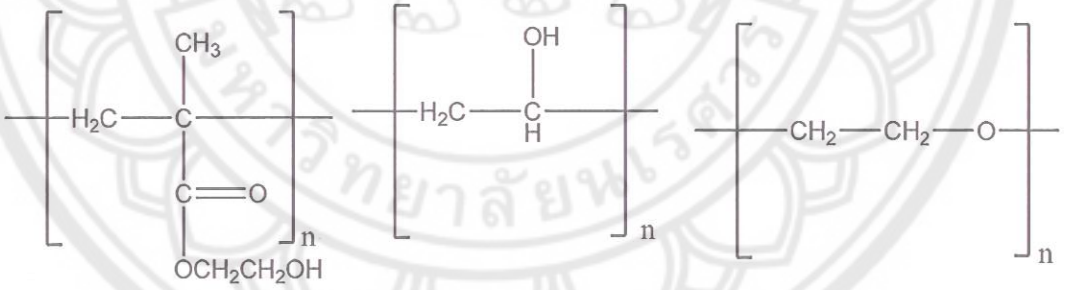
Polymer networks can be synthesized using various chemical methods (e.g., photo- and thermal-initiated polymerization). The polymer engineer can design and synthesize polymer networks with molecular-scale control over structure such as crosslinking density and with tailored properties, such as biodegradation, mechanical strength, and chemical and biological response to stimuli [45].

Neutral synthetic polymers can be generated from derivatives of poly(hydroxyethyl methacrylate) (poly(HEMA)), poly(ethylene glycol) (PEG), and poly(vinyl alcohol) (PVA) (Figure 2). PEG hydrogels are one of the most widely studied and used materials for biomedical applications. PEG hydrogels are nontoxic,

non-immunogenic, and approved by the US Food and Drug Administration for various clinical uses. In many cases, PEG has been applied as a "stealth material" since it is inert to most biological molecules such as proteins [46].

For ionic gels containing weakly acidic pendent groups, the equilibrium degree of swelling increases as the pH of the external solution increases, while the degree of swelling increases as the pH decreases for gels containing weakly basic pendent groups. Numerous properties, e.g., ionic content, ionization equilibrium considerations, nature of counterions and nature of the polymer, contribute to the swelling of ionic hydrogels, and these have been extensively studied [47-49]. Examples of some commonly studied ionic polymers include poly(acrylic acid), poly(methacrylic acid), polyacrylamide, poly(diethylaminoethyl methacrylate), and poly(dimethylaminoethyl methacrylate) (Figure 2).

Neutral polymers

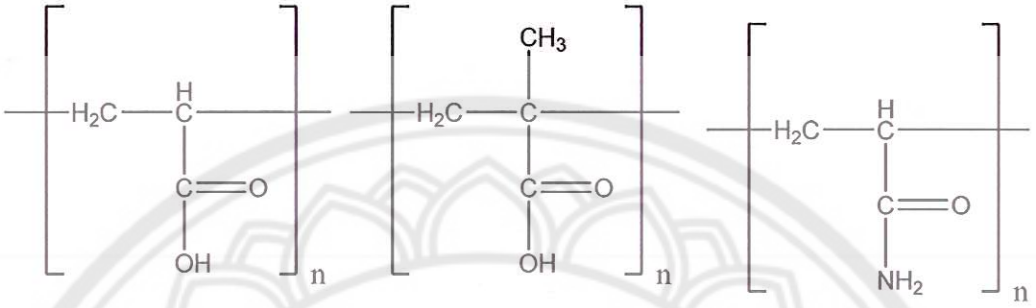


Poly(hydroxyethyl methacrylate) (PHEMA)

Poly(vinyl alcohol) (PVA)

Poly(ethylene glycol) (PEG)

Ionic polymers



Poly(acrylic acid) (PAA)

Poly(methacrylic acid) (PMMA)

Polyacrylamide (PAAm)

Figure 2 Representative chemical structures of synthetic neutral and charged polymers.

Cross-linking methods for *in situ* forming hydrogels

Physical cross-linking

Physical cross-linking between polymers can be obtained by using several non-covalent interactions, such as hydrophobic interactions, ionic interactions, hydrogen bonding, host–guest interactions or combinations of these. The most frequently exploited interactions for building physically cross-linked hydrogels are hydrophobic interactions because they are strong interactions in aqueous environment and hydrogels can simply be prepared by using amphiphilic block copolymers.

Chemical cross-linking

Chemical cross-linking yields covalent bonds between different polymer chains, and the resulting hydrogel network is in general more resistant to mechanical forces than physically cross-linked networks. Many conventional coupling reactions have been used to obtain cross-linked polymers, but in recent years especially "click chemistry" [50] (azide–alkyne cycloaddition) and also "native chemical ligation" (ligation of a *C*-terminal thioester to an *N*-terminal cysteine residue) [51] are becoming more popular due to their ease of use and high conversion.

Hydrogel based on natural polymers

Many hydrogels with natural polymers as building blocks have been recently developed. These natural polymer networks display multiple advantages over synthetic polymer gels for biomedical applications with respect to their often inherent biocompatibility, biodegradability, and good cell adhesion properties. Biopolymer-based hydrogels have been investigated extensively for cell encapsulation for regenerative medicine. The natural extracellular matrix has more in common with these biopolymer gels as compared to synthetic polymer hydrogels, generally resulting in better cell survival and differentiation. Besides cell encapsulation, also growth factors are often incorporated to enhance the performance of these artificial tissues. This chapter mainly focuses on the protein and/or growth factor release from hydrogels based on natural polymers used for tissue engineering applications.

Polysaccharide

Polysaccharides are in general very hydrophilic polymers and are therefore very suitable for the design of hydrogels. The most commonly used polysaccharides in recent hydrogel research aimed at protein delivery are chitosan, alginate, hyaluronic acid and dextran (Figure 3) [52, 53]. Hydrogels based on these polysaccharides are discussed below with respect to their use for protein delivery. Besides other polysaccharides such as cellulose, heparin, and pullulan have also been studied for the development of hydrogels.

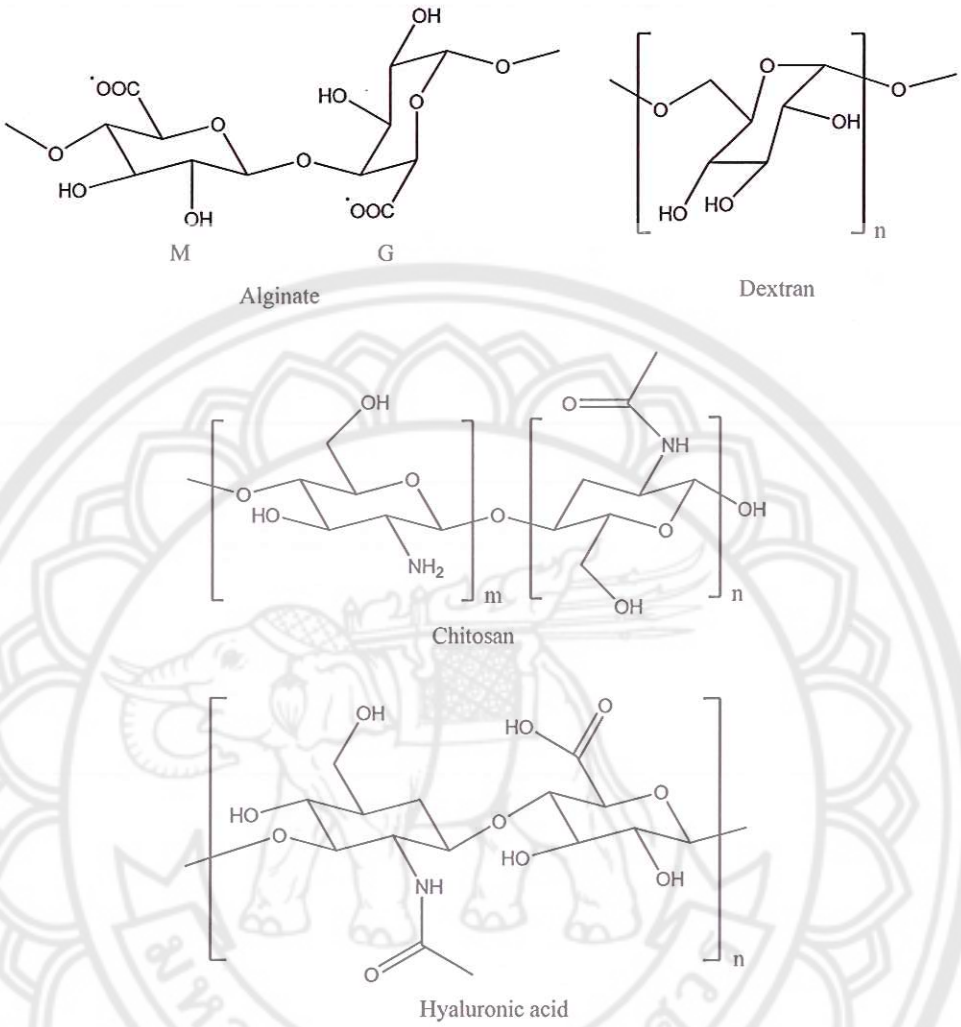


Figure 3 Most commonly used polysaccharide for hydrogel preparation for biomedical applications (M = manuronic acid and G = guluronic acid)

Dextran

Dextran is a hydrophilic polysaccharide that consists of α-1,6-linked D-glucopyranoses with some degree of 1,3-branching. Several methods have been exploited to crosslink dextran to obtain hydrogels [54]. Besides for the design of hydrogels, dextran has also been used as a carrier system for many therapeutic and contrast agents. The high number of available hydroxy groups presents many options for derivatization of dextran for subsequent physical or chemical cross-linking.

Hyaluronic acid

Hyaluronan or hyaluronic acid (HA) (Figure 3) is a linear glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetylglucosamine [55, 56]. HA is a negatively charged, naturally occurring polysaccharide with high molecular weights up to 10^7 Da. It is found mainly in the extracellular matrix (ECM) and in the synovial fluids of joints, where it reduces the friction of bones due to its unique viscoelastic properties [57]. Because of HA's biocompatible and attractive physical properties, biomaterials based on HA have mainly been developed for tissue engineering purposes. The delivery of proteins such as growth hormones is another important issue. A few HA gels have been studied for their protein delivery possibilities including photopolymerized HA, HA–tyramine conjugates cross-linked using an oxidation reaction, HA–SH cross-linked using disulfide bond formation, and HA cross-linked by Michael addition [58, 59]. For the preparation of gel networks, usually the carboxylic groups of hyaluronan are derivatized to obtain cross-linking functionalities in the polymer chains. Since HA is negatively charged at physiological pH, the protein release rate will be affected by the charge of the protein. Nevertheless, complete release can be obtained by enzymatic degradation of HA by hyaluronidase, which is present in biological tissues.

Chitin-Chitosan

Chitin is the most abundant natural amino polysaccharide and is estimated to be produced annually almost as much as cellulose. It has become of great interest not only as an underutilized resource, but also as a new functional material of high potential in various fields. Chitin, a naturally abundant mucopolysaccharide, and the supporting material of crustacean, insect, etc., is well known to consist of 2-acetamido-2-deoxy-β-D-glucose through a β(1 → 4) linkage. Chitin can be degraded by chitinase. Its immunogenicity is exceptionally low, in spite of the presence of nitrogen. It is a highly insoluble material resembling cellulose in its solubility. It may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group. Like cellulose, it functions naturally as a structural polysaccharide. Chitin is a

white, hard, inelastic, nitrogenous polysaccharide and the major source of surface pollution in coastal areas [16].

Chitosan is the partially *N*-acetylated derivative of chitin. The structures of celluloses, chitin and chitosan are shown in Figure 4. Chitin and chitosan are of commercial interest due to their high percentage of nitrogen (6.89%) compared to synthetically substituted cellulosed (1.25%), which makes chitin useful as a chelating agent. Chitin and chitosan are recommended as suitable functional materials, because they have many intriguing properties such as biocompatibility, biodegradability, non-toxicity, adsorption properties.

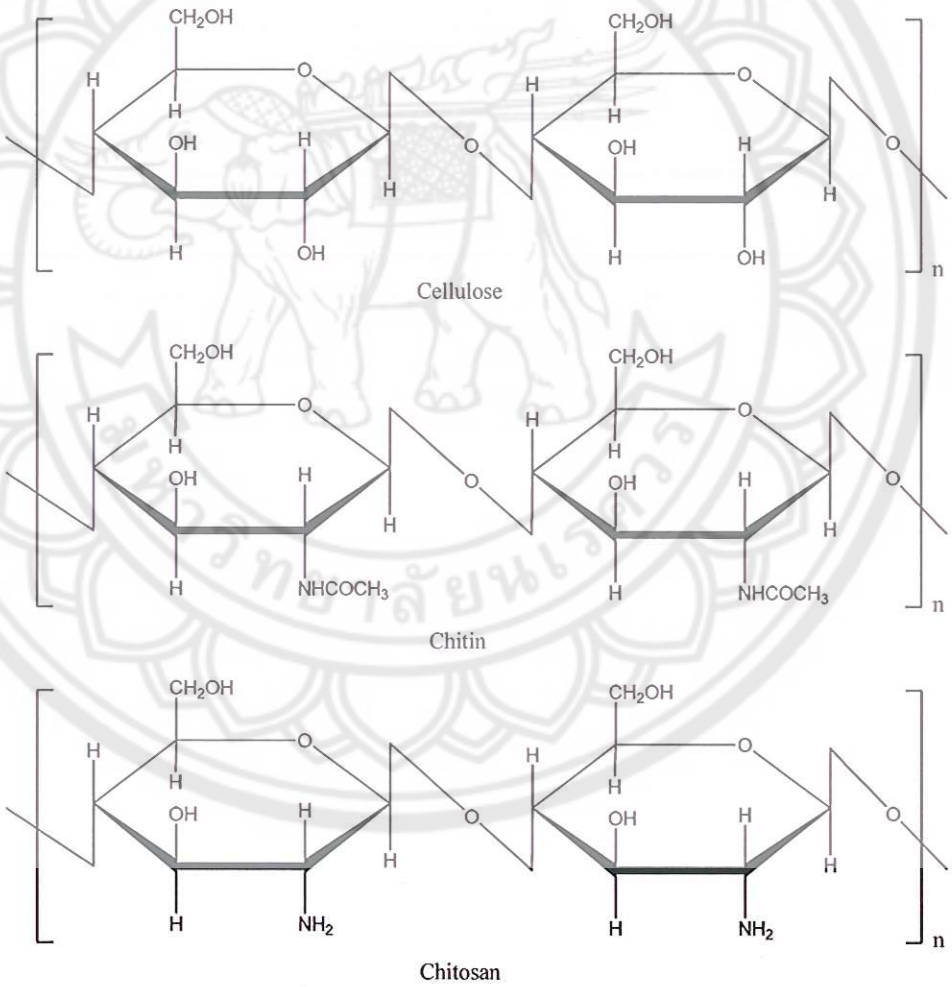


Figure 4 Chemical structures of cellulose, chitin and chitosan.

Carboxymethylchitosan (CMC)

Deacetylation of chitin produces chitosan, a polymer widely studied for its pharmaceutical and non-pharmaceutical applications. Because chitin shows the hurdle in comprehending these applications due to its limited solubility, CMC helps to improve its solubility in water.

Chitosan, a cationic copolymer of glucosamine and *N*-acetylglucosamine, is a partially deacetylated derivative of chitin. Chitosan has a unique set of useful characteristics such as biorenewability, biodegradability, biocompatibility, bio-adhesively and nontoxicity. Chitosan and its derivatives are used in various fields: pharmaceutical, biomedicine [60], water treatment [61], cosmetics [62], agriculture[63] and food industry [64]. However the applications of chitosan suffer severe limitations since it is insoluble in neutral or alkaline pH because of its very stable crystalline structure arising from string hydrogen bonds. The solubility is observed only in acidic aqueous solutions below pH 6.5 (below the pKa of chitosan). The solubility of chitosan can be improved by depolymerization and its chemical modifications. Chitosan has reactive amino, primary hydroxyl and secondary hydroxyl group which can be used for chemical modifications under mild reaction conditions to alter its properties (Figure 4) [65]. Many water-soluble derivatives have been prepared by quaternarization or by introducing hydrophilic groups, e.g. hydroxypropyl, dihydroxyethyl, hydroxyalkylamino, sulfate, phosphate, or carboxyalkyl groups as carboxymethyl, carboxyethyl, carboxybutyl or by grafting water-soluble polymers in the macromolecular chain of chitosan [66]. Compared with other water-soluble chitosan derivatives, CMC has been widely studied because of its ease of synthesis, ampholytic character and possibilities of ample of applications.

Physicochemical properties of CMC

Solubility and aggregation

A significant characteristic of CMC is its solubility in water. Compared with chitosan, the solubility of CMC in aqueous solution is improved remarkably because of the introduction of carboxymethyl groups. The aggregation behavior is seen in dilute aqueous solution of *O*-carboxymethylchitosan (OCMC) [67]. The combined driving forces that make OCMC soluble in water include the H-bonding between water and the polymer and presence of COO^- on the OCMC chain whereas the intermolecular H-bonding of OCMC and the electrostatic repulsion between them are the main driving forces for OCMC aggregation in solution. The aggregation is dominated by interchain aggregation, with the glucose backbones of OCMC forming the hydrophobic domains, and the dissociated carboxylic groups and the hydrophilic groups around the backbone forming the hydrophilic ones.

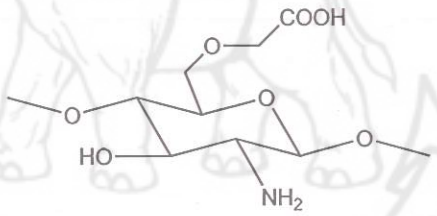


Figure 5 Chemical structure of *O*-carboxymethylchitosan (OCMC)

Moisture retention and absorption properties of CMC

The moisture retention properties of CMC have received considerable attention for its possible use in cosmetics and clinical medicine. In 2003, Lingyun Chen [68, 69] has demonstrated that the moisture absorption and retention abilities of CMC are closely related to the degrees of deacetylation (DD 28–95%) and degrees of substitution (DS 0.15–1.21) values. The 6-carboxymethyl groups in the molecular structure of chitin and chitosan was a main active site responsible for moisture retention. Moisture-retention abilities were also related to molecular weight that higher molecular weight of CMC improved its moisture-retention abilities.

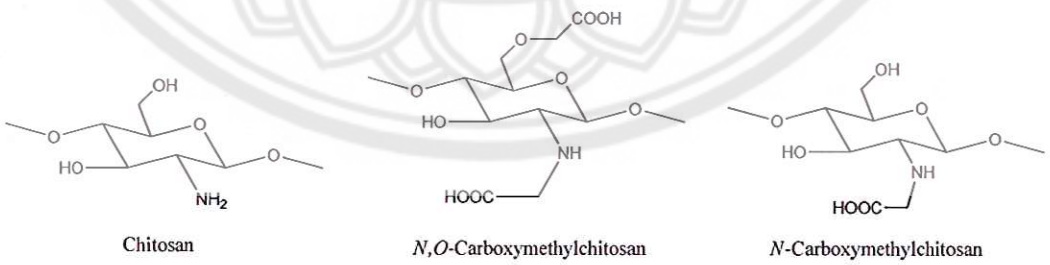
Biological properties of CMC

Antioxidant activity

The antioxidant activity of chitosan and its derivatives has indicated that the active hydroxyl and amino group in the polymer chains may take a part in free radical scavenging and contributed to the antioxidant activity. The contents of active hydroxyl, amino, amido groups in their polymer chains as well as molecular weight affect the antioxidant activity of chitosan and derivatives [70, 71].

Antimicrobial activity

In 2001, Xiao Fei Liu was demonstrated that chitosan inhibits the growth of varieties of bacteria and fungi [72, 73]. The antimicrobial activity of chitosan is influenced by its molecular weight, degree of acetylation, concentration in solution and pH of the medium. The antibacterial activities were found to increase in the order of NOCMC<chitosan<OCMC (Figure 6). The reason is the dependence of polycation's antibacterial action on effective number of $-NH_3^+$ group. In NOCMC, the effective number of $-NH_3^+$ is lower because of $-CH_2COOH$ substitution leading to decrease in antibacterial activity. In OCMC, substitution occurred only at –OH, hence its number of $-NH_2$ was not changed. Moreover, COOH group in OCMC may have reacted with the $-NH_2$ group intra- or intermolecularly and charged these $-NH_2$ groups. So, in the same condition, the number of $-NH_3^+$ groups of OCMC was more than that of chitosan. Therefore, the antibacterial activity of OCMC increased.



Chitosan *N,O*-Carboxymethylchitosan *N*-Carboxymethylchitosan

Figure 6 Chemical structure of chitosan, *N,O*-carboxymethylchitosan (NOCMC) and *N*-carboxymethylchitosan (NCMC) Applications of CMC Targeted drug delivery

Fe_3O_4 nanoparticles have attracted scientists due to their multifunctional properties such as small size, superparamagnetism and low toxicity. However, Fe_3O_4 nanoparticles tend to aggregate due to strong magnetic dipole-dipole attractions between particles and need stabilization by surfactants, oxides or polymers. A Fe_3O_4 nanoparticle coated with polymers are composed of the magnetic cores to provide favorable functional groups and features, which have various applications for drug delivery systems, therapeutic regimes, cell/enzyme immobilization and diagnostic magnetic resonance imaging. Chitosan is one such polymer being used for this purpose. However, chitosan-magnetic composites were either aggregates or unstable due to polymer cross-linking or physi-sorption and furthermore chitosan has no suitable functional groups to bind directly on to Fe_3O_4 nanoparticles. CMC fulfill these requirements. In addition, using the carboxylic moiety as a binding site, various molecules (e.g., DNA, protein) and antibody, could also be loaded onto the OCMC/ Fe_3O_4 nanoparticles for the magnetically targeted delivery [74].

Cosmetics

CMC and chitin are used more and more widely in cosmetics and as soft tissue augmentation in medicine for their excellence moisture-retention ability [75, 76]. In 1996, Lapasin [75, 76] demonstrated that NCMC solution (1.0%) at pH 4.80 is a valuable functional ingredient of cosmetic hydrating creams in view of its durable moisturizing effect in the skin. The film-forming ability of NCMC assists in imparting a pleasant feeling of smoothness to the skin and in protecting it from adverse environmental conditions and consequences of the use of detergents.

Smart hydrogels

Smart hydrogels are defined as materials able to undergo transitional changes in response to environmental stimuli [77]. They can rather abruptly swell, shrink, degrade, or undergo a sol–gel phase transition when exposed to external physical or chemical triggers such as changes in pH, temperature, solvent, pressure, ionic strength, light, and concentration of specific biomolecules [78, 79]. Environmental triggers can be exploited to accomplish specific functions such as drug release, protein separation and muscle activity, or to design *in situ* gelling systems.

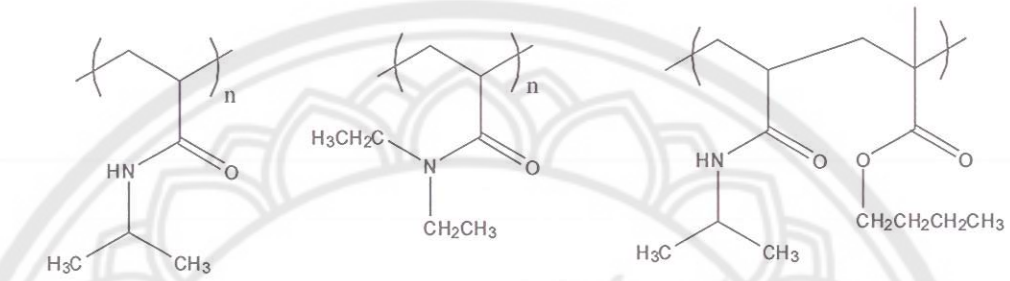
Stimuli-sensitive polymers

Stimuli sensitivity has been widely applied for the design of injectable *in situ* forming hydrogels, with pH and temperature responsive systems being the most attractive representatives. In the past 10–15 years, research has shifted its interest from the area of implantable materials to the fast-developing field of injectable *in situ* gelling systems. *In situ* forming hydrogels exist as viscous but still liquid aqueous formulations prior to administration but abruptly turn into gels upon administration [80, 81]. In contrast to permanent networks formed by chemical cross-linking, stimuli-sensitive hydrogels are transient physical networks that can be reversibly transformed into solutions by varying the environmental conditions. The advantages of these delivery systems, able to form macroscopic drug-encapsulating gels at the site of injection, include improved patient compliance, cost reduction compared to surgical intervention, and the ability to overcome the limitations associated with drug post-loading techniques.

Temperature-sensitive hydrogel

Temperature-sensitive hydrogels are the most commonly studied class of environmentally sensitive polymer systems in drug delivery research [82]. Many polymers exhibit a temperature-responsive phase transition property and the structures of some of those polymers are shown in Figure 7. The common characteristic of temperature-sensitive polymers is the presence of hydrophobic groups such as methyl, ethyl and propyl groups. Of the many temperature-sensitive polymers, poly(*N*-isopropylacrylamide) poly(NIPAAm) is probably the most extensively studied.

Poly(*N,N*-diethylacrylamide (Poly(DEAAm)) is also widely investigated because of its lower critical solution temperature (LCST) in the range of 25–32 °C, close to the body temperature (37°C). Copolymers of NIPAAm can also be made using other monomers, e.g. butylmethacrylate (BMA), to alter the LCST.



Poly(*N*-isopropylacrylamide) (Poly(NIPAAm))

Poly(*N,N*-diethylacrylamide) Poly(DEAAm)

Poly(NIPAAm-*co*-BMA)

Figure 7 Chemical structures of some temperature-sensitive polymers

Properties of temperature-sensitive hydrogels

Most polymers increase their water-solubility as the temperature increases. Polymers with LCST, however, decrease their water-solubility as the temperature increases. Hydrogels made of LCST polymers shrink as the temperature increases above the LCST. This type of swelling behavior is known as inverse (or negative) temperature-dependence. The inverse temperature-dependent hydrogels are made of polymer chains that either possess moderately hydrophobic groups or contain a mixture of hydrophilic and hydrophobic segments. It should be mentioned that if the polymer chains are too hydrophobic, they would not dissolve in water at all. At lower temperatures, hydrogen bonding between hydrophilic segments of the polymer chain and water molecules dominates, leading to enhanced dissolution in water. As the temperature increases, however, hydrophobic interactions among hydrophobic segments become strengthened, while hydrogen bonding becomes weaker. The net result is shrinking of the hydrogels due to inter-polymer chain association through hydrophobic interactions. In general, as the polymer chain contains more hydrophobic constituent, LCST becomes lower. The LCST can be changed by adjusting the ratio of hydrophilic and hydrophobic segment of the polymer. One approach is to make

copolymers of hydrophobic (e.g. NIPAAm) and hydrophilic (e.g. acrylic acid) monomers[83]. The continuous phase transition of poly(NIPAAm) is known to be changed to a discontinuous one by incorporating a small amount of ionizable groups into the gel network or by changing solvent composition [84]. Copolymerization of NIPAAm with different types of monomers results in hydrogels with more versatile properties, such as faster rates of shrinking when heated through the LCST, and sensitivity to additional stimuli [85].

In 2007, Zhou and co-worker [86] synthesized a novel aqueous thermo-responsive adsorbent material for DNA deposition, which was a star-shaped copolymer with 4-branched chains. Each chain composed of a cationic poly(*N,N*-dimethylaminopropyl acrylamide) (poly(DMAPAAm)) which formed an inner domain for DNA binding and a thermo-responsive poly(NIPAAm) block, which formed an outer domain for surface adsorption. Complex formation between the copolymer and the luciferase-encoding plasmid DNA occurred immediately upon simple mixing in an aqueous medium. The luciferase activity observed was higher than that observed on a DNA-coated substrate with or without the cationic polymer before and after complete adhesion and by conventional solution transfection using the polyplexes. The activity was enhanced with an increase in the charge ratio (surfactant/pDNA) with permissible cellular cytotoxicity.

In 2008, Jones and co-worker [87] synthesized poly(2-(hydroxyethyl)methacrylate) (poly(HEMA)), poly(4-(hydroxybutyl)methacrylate) (poly(HBMA)), poly(6-(hydroxyhexyl)methacrylate) (poly(HHMA)), poly(NIPAAm), and the copolymers composed of *N*-isopropylacrylamide (NIPAAm) and methacrylic acid (MA) by free radical polymerization. Thermo-responsive behavior was only observed with hydrogels composed of HEMA, MA, and NIPAAm in which pulsatile drug release was obtained by elevating the temperature from below to above the LCST. A greater mass and enhanced pulsatile release of drug, with the associated greater antimicrobial properties (an 10^8 reduction in viability of *Staphylococcus* epidermidis in 15 min), was associated with poly(NIPAAm-co-HEMA)(1:1 molar ratio). It is suggested that the pulsatile drug release and favorable antimicrobial and mechanical properties of candidate hydrogels, particularly poly(HEMA-co-NIPAAm), offer promise as thermo-responsive, antimicrobial biomaterials that may be used as wound dressings, medical implants, or coatings of medical devices.

In 2009, Hou-feng Zhang and co-worker [88] synthesized a novel type of hydrogel by graft copolymerization of NIPAAm and CMC. The poly(NIPAAm) grafted with CMC hydrogel has a better temperature sensitivity and swelling ratio, compared to the poly(NIPAAm) hydrogel. This thermo-sensitive and biodegradable hydrogel may have the potential applications in controlled drug delivery system. They can also be used to separate and purify some biological materials such as proteins, enzymes and amylose.

In 2010, Ma and co-worker [89] synthesized a novel biodegradable monomer, methacrylate-polylactide (MAPLA), and it was then copolymerized with NIPAAm and HEMA to develop bioabsorbable and thermally responsive hydrogels. Poly(NIPAAm-*co*-HEMA-*co*-MAPLA) formed from three monomer feed ratios, 84/10/6, 82/10/8, and 80/10/10 were synthesized, with a higher MAPLA feed ratio giving rise to a lower LCST, higher mechanical strength, and faster degradation speed. All three of the hydrogels had LCSTs below the body temperature and formed mechanically strong hydrogels at 37 °C. These hydrogels, upon cleavage of PLA residues by hydrolysis, became completely soluble at 37 °C and exhibited no cytotoxicity associated with degradation products. This novel hydrogel design represents an injectable biomaterial that is suitable for mechanical support applications in regenerative medicine, such as for ventricular bulking following myocardial infarction.

pH-sensitive hydrogel

All the pH-sensitive polymers contain pendant acidic (e.g. carboxylic and sulfonic acids) or basic (e.g. ammonium salts) groups that either accept or release protons in response to changes in environmental pH. The polymers with a large number of ionizable groups are known as polyelectrolytes. Figure 8 shows chemical structures of some examples of anionic and cationic polyelectrolytes and their pH-dependent ionization. Poly(acrylic acid) (poly(AA)) becomes ionized at high pH, while poly(N,N′-diethylaminoethyl methacrylate) (poly(DEAEMA)) becomes ionized at low pH. As shown in Figure 8, cationic polyelectrolytes, such as poly(DEAEMA), dissolve more, or swell more if crosslinked, at low pH due to ionization. On the other hand, polyanions, such as poly(AA), dissolve more at high pH [6].

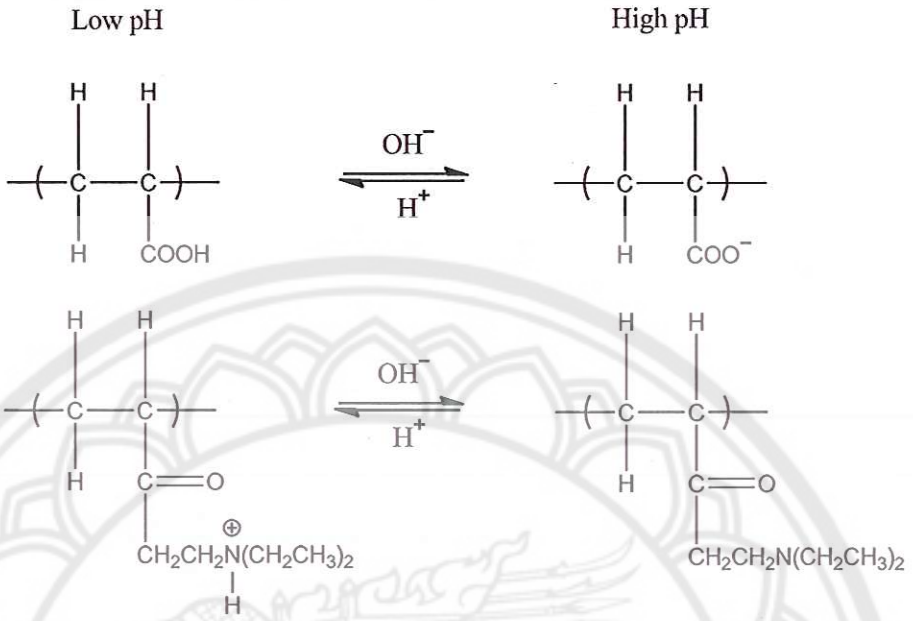


Figure 8 pH-dependent ionization of polyelectrolytes. Poly(acrylic acid) (top) and poly(N,N′-diethylaminoethyl methacrylate) (bottom).

Properties of pH-sensitive hydrogels

Hydrogels made of crosslinked polyelectrolytes display significant differences in swelling properties depending on the pH of its environment. The pendant acidic or basic groups on polyelectrolytes undergo ionization just like acidic or basic groups of monoacids or monobases. Ionization on polyelectrolytes, however, is more difficult due to electrostatic effects exerted by other adjacent ionized groups. This tends to make the apparent dissociation constant (K_a) different from that of the corresponding monoacid or monobase. The presence of ionizable groups on polymer chains results in swelling of the hydrogels much beyond that can be achievable by nonelectrolyte polymer hydrogels. Since the swelling of polyelectrolyte hydrogels is mainly due to the electrostatic repulsion among charges present on the polymer chain, the extent of swelling is influenced by any condition that reduce electrostatic repulsion, such as pH, ionic strength, and type of counter ions [90]. The swelling and pH-responsiveness of polyelectrolyte hydrogels can be adjusted by using neutral comonomers, such as 2-hydroxyethyl methacrylate, methyl methacrylate and maleic anhydride [91, 92]. Different co-monomers provide different hydrophobicity to the polymer chain, leading

to different pH-sensitive behavior. Hydrogels made of poly(methacrylic acid) (Poly(MA)) grafted with poly(ethylene glycol) (PEG) have unique pH-sensitive properties[93]. At low pH, the acidic protons of the carboxyl groups of (Poly(MA)) interact with the ether oxygen of PEG through hydrogen bonding, and such complexation results in shrinkage of the hydrogels. As the carboxyl groups of (Poly(MA)) become ionized at high pH, the resulting decomplexation leads to swelling of the hydrogels. The same principle can be applied to IPN systems where two different types of polymer chain interact through pH-dependent hydrogen bonding.

In 2006, Xiangchun Yin [94] demonstrated the synthesis of temperature and pH-sensitive random copolymers of *N*-isopropylacrylamide (NIPAAm) and propylacrylic acid (PAA) *via* the reversible addition fragmentation chain transfer (RAFT). These copolymers exhibited temperature-induced phase transition behavior over small pH ranges. The polar character of the PAA units changed from strongly hydrophobic when it was protonated at slightly acidic conditions to highly hydrophilic upon ionization, leading to copolymers which their polarity and solubility were very sensitive to small environmental pH changes.

In 2007, Shun Wan and co-worker [95] demonstrated the synthesis of thermo- and pH-responsive micellization of *N*-isopropylacrylamide (NIPAAm) and acrylic acid (AA) from hydroxyethyl cellulose (HEC) backbone *via* successive radical polymerization. These copolymers were capable of self-assembling into three forms of nanosize micellar structures driven by external pH and temperature changes. In this process, hydrogen-bonding interaction of the complementary grafts, poly(NIPAAm) and poly(AA), and hydrophobic aggregation of poly(NIPAAm) played a crucial role. Because of pH changes, the micelles with poly(NIPAAm)/poly(AA) complex as the core and solvated HEC as the shell can be obtained below pH 4.6. These thermo-induced micelles can further respond to pH change, resulting in the formation of the micelles with a three-layer structure.

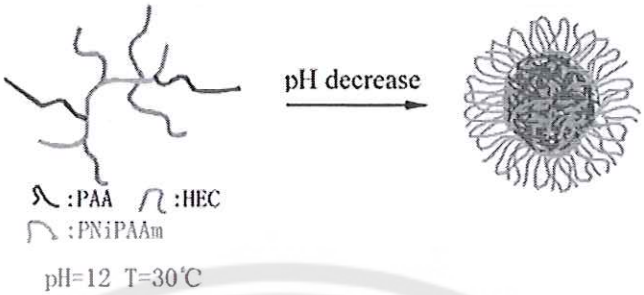


Figure 9 A proposed scheme of the formation of pH-induced micelles of copolymer in water [95]

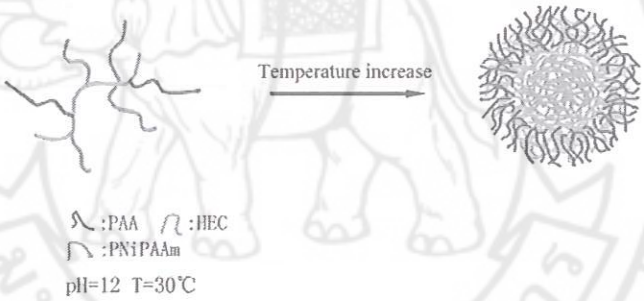


Figure 10 A proposed scheme of the formation of temperature-induced micelles of copolymer at pH 12 [95]

Other stimuli-sensitive polymers

Biomolecule-sensitive hydrogel

On-demand release of drugs is particularly relevant for drugs that necessitate a more complex release profile able to mimic varying physiological concentrations over time (e.g., insulin or hormones). Glucose-sensitive hydrogels are insulin reservoirs of polymeric networks that ideally release the drug on demand when the glucose

concentration exceeds a certain level. One strategy to achieve this goal relies on the use of pH-responsive hydrogels entrapping glucose oxidase, catalase, and insulin. *N,N*-dimethylaminoethyl methacrylate (DMAEMA, pK_a 8.4) [96] has often been introduced into copolymer hydrogels to render them pH-sensitive. When glucose diffuses into the hydrogels, it is converted to gluconic acid due to the action of glucose oxidase. The formed gluconic acid causes a pH drop, responsible for the protonation of DMAEMA groups and swelling of the hydrogel due to increased electrostatic chain repulsions, resulting in larger pores in the gels and release of insulin[97]. A similar approach was reported using a sulfonamide-based glucose-responsive hydrogel[98]. Kitano and co-worker [99, 100] proposed poly(*N*-vinyl-2-pyrrolidone-co-phenylboronic acid) (poly(NVP–PBA)) as a chemically regulated delivery system for insulin. The diol moiety on PBA allows the binding of glucose followed by pulsatile insulin release.

Nanogel

Nanogels possess stimuli responsive behavior, regarded as smart hydrogels, such as thermo- and pH-sensitivity [101]. Many nanoparticulate systems with sizes between 10 and 1000 nm (but ideally <200 nm) such as nanocapsules, polymeric micelles, liposomes, and dendrimers have been developed for drug delivery applications [102, 103]. Because of their nano-scale size, nanoparticles are able to circulate in the bloodstream, depending on their size and surface properties, for a couple of hours and overcome certain anatomical barriers. Besides, they can also reach tumor tissues, due to the enhanced permeation and retention (EPR) effect and coupling of targeting ligands on their surface aims for cellular recognition/internalization to increase efficacy of, for example, anticancer drugs loaded in such particles [104]. Nanogels are a relatively new class of nanoparticulate carriers that have been shown to deliver drugs intracellularly by different cellular uptake mechanisms (clathrin- and caveoli-mediated endocytosis, pinocytosis, and phagocytosis). Because of aimed intracellular drug delivery, especially pH-sensitive nanogels are of interest due to lower pH values in lysosomes, which might trigger the release of entrapped drugs

[105]. So far, nanogels have been mainly exploited for the (targeted) delivery of low molecular weight drugs but are also under investigation for the release of nucleic acid based drugs and pharmaceutical proteins [106].

CHAPTER III

RESEARCH METHODOLOGY

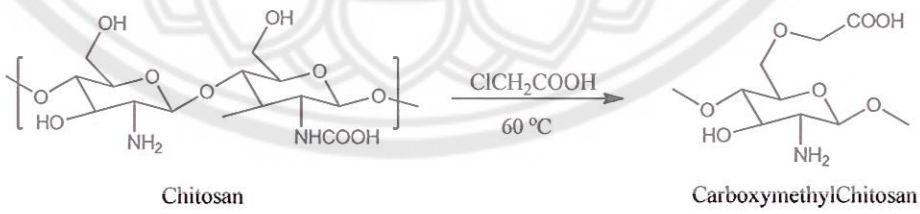
Materials

Chitosan from crab (M_n = 1.4×10^5 g/mol) (Taming Enterprise, 98% deacetylation) was used without purification. *N*-isopropylacrylamide (NIPAAm) (Acros, 99%) was recrystallized in hexane before used to remove inhibitors. Acrylic acid (AA) (Acros, 99.5%) was distilled under reduced pressure before used. 1,6-Hexamethylene diisocyanate (HDI)(Carlo Erba, 99%), diammonium peroxodisulphate (APS) (Carlo Erba, 98%), sodium metabisulfate ($Na_2S_2O_4$) (Carlo Erba, 97%) and indomethacin (Sigma, 90%) were used as received. All other chemicals were analytical-grade and used as without purification.

Syntheses

1. Synthesis of carboxymethylchitosan (CMC) from chitosan

Chitosan (40 g) swollen in isopropanol (100 ml) for 24 h was reacted with a NaOH solution at room temperature for 75 min, and followed by the reaction with monochloroacetic acid (40 g, 0.51mol in H_2O 100 ml) at 60 °C for 5 h. The solution was then precipitated in an excess of methanol. To remove salts, it was washed with a methanol:H_2O solution (70:30 v/v). CMC was filtered and dried at 40 °C of 24 h.



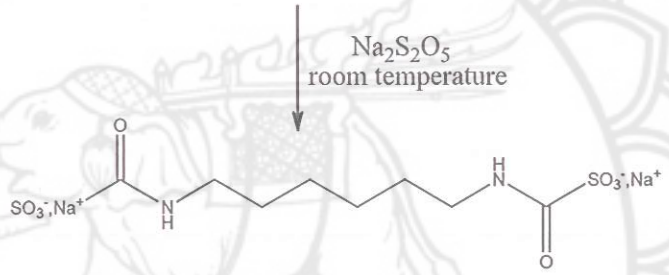
Chitosan CarboxymethylChitosan

Scheme 1 Carboxymethylation of chitosan to form CMC

2. Synthesis of hexamethylene-1,6-di-(aminocarboxysulfonate) (HDA) as a water soluble crosslinker

1,6-Hexamethylene diisocyanate (HDI) (6.73 g, 0.02 mol) was introduced into a sodium metabisulfate solution (8.36 g, 0.04 mol in 15 ml of H_2O) and the mixture was then stirred at room temperature for 24 h. The product was precipitated in an excess of acetone, which was then filtered and dried *in vacuo*.

Hexamethylene diisocyanate (HDI)



Hexamethylene-1,6-di-(aminocarboxysulfonate) (HDA)

Scheme 2 Synthesis of hexamethylene-1,6-di-(aminocarboxysulfonate) (HDA)

3. Synthesis of poly(NIPAAm-co-AA) -grafted with CMC hydrogels

An example for the synthesis of CNPA2 in Table 1 is illustrated. Other CMC hydrogels were prepared in a similar fashion with proper amounts of reagents used. CMC (0.5 g, 0.0023 mol of carboxymethyl glucosamine unit) and NIPAAm (0.5 g, 0.0044 mol) were dissolved in DI water (10 ml) with stirring under N_2 for 30 min at room temperature. After heating to 60°C, APS (0.0025 g 0.0089 mol), a radical initiator, was added to the solution, which was then stirred for 45 min. AA (0.16 g, 0.0022 mol) was then added as a co-monomer into the solution with stirring for 4 h. HDA (0.05g, 0.0001 mol), a crosslinker, was introduced into the solution with continuously stirring for another 30 min. After reaction completed, the solution was dried *in vacuo* at 40 °C for 24 h to form the hydrogel. To remove ungrafted poly(NIPAAm) and poly(AA), the hydrogel was immersed in excess acetone for 24 h,

filtered and dried. It should be noted that ungrafted poly(NIPAAm) and poly(AA) were well soluble in acetone but the covalently crosslinked poly(NIPAAm-*co*-AA) in CMC hydrogel is not.

Table 1 Feed composition for the preparation of copolymer hydrogels

Sample	CMC (g)	NIPAAm (g)	AA (g)	molar ratio of CMGA[a]:NIPAAm:AA	%G[b]	%GE[c]
CMC	0.5	-	-	-	-	-
CNPA0	0.5	0.5	-	1:2:0	47.05 ± 0.19	47.05 ± 0.19
CNPA1	0.5	0.5	0.08	1:2:0.5	100.21 ± 1.15	86.39 ± 0.99
CNPA2	0.5	0.5	0.16	1:2:1	118.77 ± 1.70	89.98 ± 1.29

[a] CMGA is carboxymethylglucosamine unit in CMC

[b] %G is grafting percentage

[c] %GE is grafting efficiency

Characterization of the polymers and hydrogels

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) was recorded on a bruker NMR spectroscopy operating at 400 MHz. Fourier transformed infrared spectroscopy (FTIR) was conducted on a Perkin-Elmer Model 1600 series FTIR spectrophotometer using KBr pellets. Morphological studies of the sample surface were carried out through LEO 1455 VP scanning electron microscopy (SEM) with an accelerating voltage of 5 kV. To prepare the hydrogel for SEM experiments, it was swollen in water at 10°C for 24 h and then lyophilized. The dried film was cut into 1×1 cm^2 in size, adhered onto an aluminum stub and coated with gold. Grafting

percentage (%G) and efficiency (%GE) were estimated by the difference of the weights before and after grafting reactions. They were calculated according to the following equations:

$$\text{Grafting percentage (\%G)} = (W_g - W_c)/W_c \times 100\% \quad (1)$$

$$\text{Grafting efficiency (\%GE)} = (W_g - W_c)/W_m \times 100\% \quad (2)$$

where W_g, W_c and W_m are the weights of dried polymer-grafted CMC, CMC and monomers (NIPAAm and/or AA), respectively. An example of the calculation of %G and % GE is illustrated in the supporting information.

Water contact angle measurement

Contact angles (θ) between water and sample films were investigated using the sessile method on a ramé-hart Model 200 Standard Contact Angle Goniometer at room temperature. A drop of water was carefully applied on a sample film and the contact angle was quickly measured before it started to swell. The reported values are the average of five different measurements.

Determination of percent crosslinking

The dried films with the dimension of 1×1 cm^2 were immerged into DI water and stirred at room temperature for 24 h to dissolve uncrosslinked portions in the hydrogel. The insoluble hydrogel was filtered and thoroughly washed with distilled water and acetone to further remove untrapped portions. The swollen gels were then dried at 30 °C for 24 h. Percent crosslinking was calculated as following:

$$\text{Percent crosslinking} = \frac{W_2}{W_1} \times 100 \quad (3)$$

where W_1 and W_2 are the weights of dried samples before and after dissolutions, respectively. The reported values are the average of at least three different measurements.

Determination of water swelling behavior

Equilibrium water content (%EWC) of the hydrogels was investigated by immersing the dried films in an aqueous solution at a given temperature and pH. The swollen films were periodically removed from the solution and excess water on their surface was wiped off. %EWC was calculated from the following equation;

$$EWC\ (\%) = \frac{W_s - W_d}{W_d} \times 100 \quad (4)$$

where W_d and W_s are the weights of dried and swollen samples, respectively.

Determination of entrapment efficiencies (%EE) and drug loading efficiencies (%DLE)

The dried hydrogels were immersed in an indomethacin-ethanol solution (0.065 mg of indomethacin in 10 ml of ethanol) at 10°C for 2 days to fully swell the drug into the hydrogels. The difference of the weights of indomethacin in the solutions before and after the swelling experiments was determined by UV-visible spectrophotometry at wavelength of 320 nm and this result reflected the weight of the entrapped drug in the hydrogel. Therefore, %EE and %DLE were calculated using the following equations;

$$\%\ \text{Entrapment efficiency (\%EE)} = \frac{\text{Weight of the entrapped drug in the hydrogel}}{\text{Weight of the loaded drug}} \times 100 \quad (5)$$

$$\%\ \text{Drug loading efficiency (\%DLE)} = \frac{\text{Weight of the entrapped drug in the hydrogel}}{\text{Weight of the dried film}} \times 100 \quad (6)$$

Studies in the in vitro drug release behavior

Indomethacin releasing behavior of the drug-entrapped hydrogels was determined as a function of solution temperature and pH. To study the effect of the solution pH on drug releasing behavior, the dried films with the dimension of 1×1 cm^2 were immersed in a phosphate buffer solution (PBS) at 25°C at pH 4, 7 or 10. The similar experiments were performed in PBS at pH 7.4 at 10, 30 or 50°C to study the effect of solution temperature on drug releasing profile. The drug concentration in the releasing media was periodically determined *via* UV-vis spectrophotometry (320 nm).

Cytotoxicity

The cytotoxicity test was carried out using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) cytotoxicity assay. Cell culture experiments were carried out using mouse fibroblasts. Cell suspension of $1x10^5$ cells/ml L929 in Minimum Essential Medium (MEM) was seeded in a 96-well plate and incubated at 37±1 °C with 5.0±0.1% CO_2 and 95±5% relatively humidity for 24±2 h to obtain confluent monolayers of cells prior to use. The dried hydrogels were sterilized in an autoclave at 121 °C for 15 min. A 'Thermanox' (Nunc) coverslip and a polyurethane film containing 0.1% Zinc diethyldithiocarbamate (ZDEC) were used as negative and positive control materials, respectively. After incubation, the viable cells were stained with MTT and incubated for 2 h. Then, MTT was removed and dimethylsulfoxide (DMSO) was added in each well. The absorbance was measured using a microplate reader at 570 nm.

Antibacterial activity

Antibacterial activity of the hydrogels against *S. aureus* (*S. aureus*, TISTR 1466) was evaluated using the optical density (OD) method, measured by a shake flash testing (150 rpm, 37 °C). Briefly, the dried hydrogel (0.1 g) was immerged into Mueller Hinton broth (HiMedia Laboratories Pvt. Ltd., India) medium (20 ml) containing 10^6 CFU/mL *S. aureus* and then the medium was incubated in humidified atmosphere. The suspension without the hydrogel was set as a control. During the

incubation process, the turbidity of the medium was measured at 650 nm for 12 h. The bacterial proliferation was reported in terms of the OD value. Each measurement was performed under aseptic conditions using aseptic techniques.

CHAPTER IV

RESULTS AND DISCUSSION

The main objective of this study was to prepare dual responsive CMC-based hydrogels. Thermo-sensitive poly(NIPAAm) and pH-sensitive poly(AA) were embedded into highly water-swollen CMC using a water soluble crosslinking agent (hexamethylene-1,6-di-(aminocarboxysulfonate) or HDA) to form a semi-interpenetrating polymer network (semi-IPN). The dual-responsive properties of CMC hydrogel can be used as triggering mechanisms for drug controlled release (Figure 1).

To synthesize the hydrogel, poly(NIPAAm) was first covalently grafted onto CMC chains *via* a surface-initiated radical polymerization using APS as an initiator. Amine radical (•NH-) can be formed on CMC chain, which give rise to the initiating site for poly(NIPAAm)-grafted CMC. The reaction was allowed for 30 min to reach 30% conversion of NIPAAm as determined *via* ^{1}H NMR spectroscopy (supporting information). In the case of CNPA0, the reaction was ceased at this low percent conversion to avoid a premature chain termination due to radical recombination. In the case of CNPA1 and CNPA2 (Table 1), appropriate amounts of AA monomers were sequentially added to the reaction vessels to further extend the polymer chains from the grafted poly(NIPAAm), leading to the formation of poly(NIPAAm-*co*-AA)-grafted CMC (Figure 1). After 4 h of poly(AA) polymerization, ^{1}H NMR spectroscopy indicated 80% conversion of AA (supporting information).

Grafting percentage (%G) of the polymers in the CMC chains were in the range of 47-119%, while their grafting efficiencies (%GE) ranged between 47% and 90% (Table 2). These numbers increased significantly when poly(AA) presented in the hydrogels (CNPA1 and CNPA2). This was attributed to the extension of the chain lengths of the grafted polymers on CMC when the reaction time was prolonged. Increasing AA incorporated in the reactions (CNPA2) also promoted both %G and %GE of the hydrogels.

It should be noted that poly(NIPAAm) and poly(AA) homopolymers might also be formed due to the existence of ammonium sulfate radicals in the solution, which served as free radical initiators in the solution. To form the hydrogels, these

polymers were left in the solution without extraction in order to simplify the hydrogel preparation process. These homopolymers were thus embedded in poly(NIPAAm-*co*-AA)-grafted CMC using a water-soluble HDA crosslinker to form semi-IPN. Therefore, it was envisioned that ungrafted poly(NIPAAm) and poly(AA) that might exist in the solution were physically locked in the CMC hydrogels without covalent bonding.

Functional group characterization of the hydrogels

The functional groups of the as-synthesized poly(NIPAAm-*co*-AA)-grafted CMC hydrogels were analyzed *via* FTIR (Figure 11). Poly(NIPAAm) and poly(AA) homopolymers were separately synthesized for use in the analysis of the characteristic signals of their functional groups. As compared to the spectra of the homopolymers (Figure 1b and 1c) and CMC (Figure 1a), the copolymers exhibited the characteristic absorption signals of amide functional groups of poly(NIPAAm): 1650 cm^{-1} (NH-CO- stretching), 1548 cm^{-1} (N-H bending) and 3436 cm^{-1} (N-H stretching), and the signals of carboxylic acid groups of poly(AA): 1736 cm^{-1} (HO-CO- stretching) and 3436 cm^{-1} (O-H stretching).

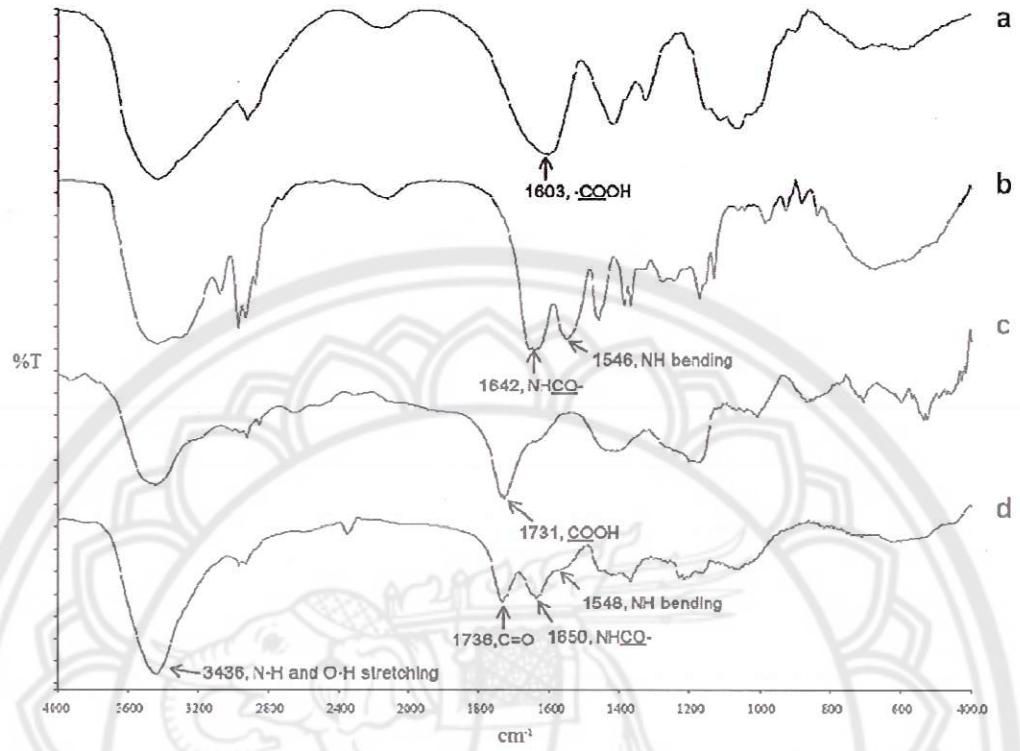


Figure 11 **FTIR spectra of (a) CMC, (b) poly(NIPAAm) homopolymer, (c) poly(AA) homopolymer and (d) poly(NIPAAm-*co*-AA)-grafted CMC hydrogel**

Determination of percent crosslinking of the hydrogels

HDA was used as a water soluble crosslinker in the formation of poly(NIPAAm-*co*-AA)-grafted CMC hydrogel. It is envisioned that poly(NIPAAm) and poly(AA) homopolymers existing in the reaction mixture were interlocked into the network, resulting in the formation of semi-IPN structure. Incorporation of these polymers in the structure tended to increase percent crosslinking of the hydrogels. This was attributed to the increase of network density due to the addition of the polymers into the structure.

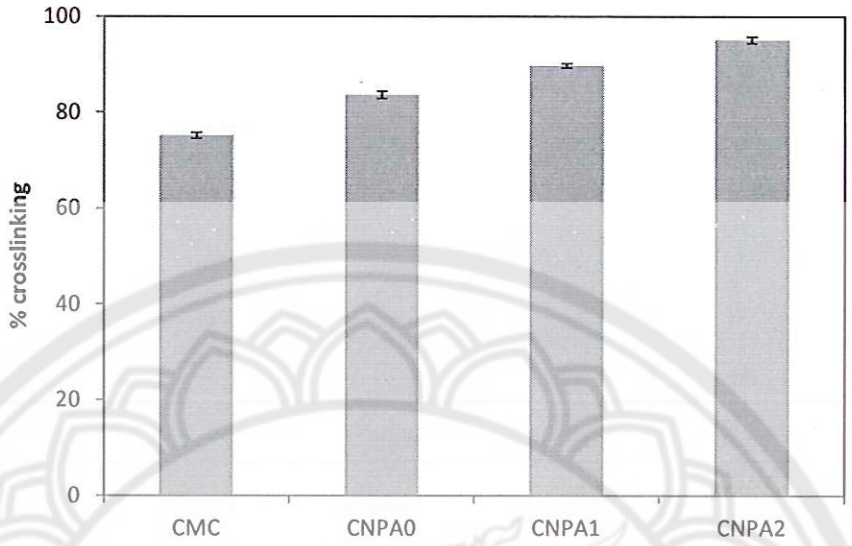


Figure 12 **Percent crosslinking of CMC and the CMC hydrogels grafted with poly(NIPAAm-*co*-AA)**

Surface morphology studies

Figure 13 illustrates the morphology studies of fully water-swollen hydrogels after lyophilization. SEM images of the crosslinked CMC without poly(AA) (CMC and CNPA0) (Figure 3A and 3B) showed dense morphologies, while those of CNPA1 and CNPA2 exhibited abundant open and porous structure. These pores existed on their surface and also inside the hydrogels (Figure 3C-3F). It was rationalized that hydrophilic poly(AA) might promote water uptake and swellability of the hydrogels, leading to the formation of micropores in the swollen structure. Increasing poly(AA) content in the hydrogels (CNPA1 as opposed to CNPA2) seemed to promote the formation of the porous structure. In good agreement with the SEM results, the CMC grafted with poly(AA) showed an increase in water swelling properties as opposed to the ones without poly(AA) and this would be discussed in details in the water swelling study section.

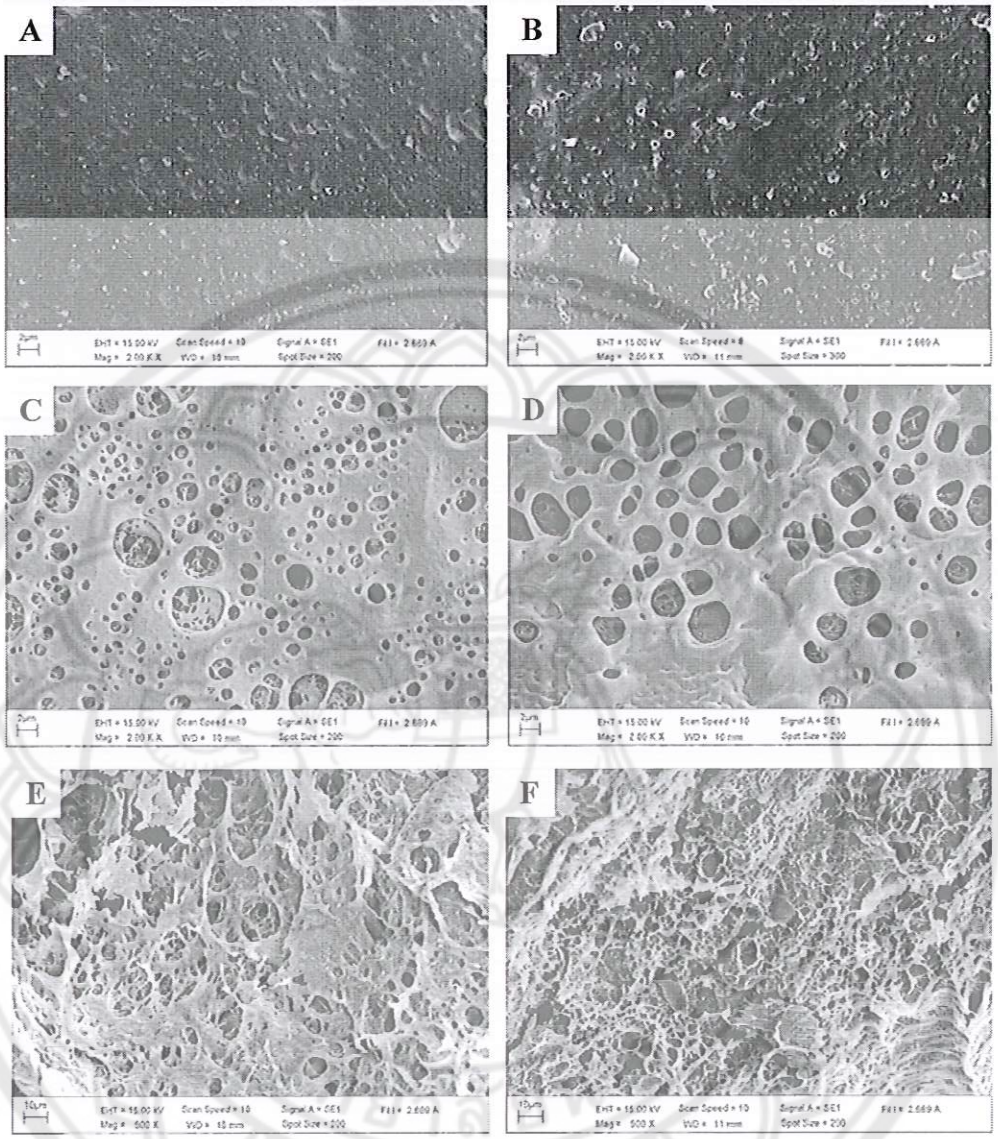


Figure 13 Surface morphology of (A) CMC, (B) CNPA0 (C) CNPA1, (D) CNPA2, and cross-sectional morphology of (E) CNPA1 and (F) CNPA2

Studies in water contact angles of the hydrogels

Effect of poly(NIPAAm) and poly(AA) grafted on CMC on surface wettability of the hydrogels was investigated by measuring their water contact angles as opposed to the unmodified CMC hydrogel. Hypothetically, increasing water contact angle implies the decrease in surface hydrophilicity of the material. According to the results in Figure 14, the increase in water contact angle from 37.6° to 45.4° was attributed to the presence of hydrophobic poly(NIPAAm) in the structure, resulting in the enhancement of surface hydrophobicity of the hydrogel. Increasing poly(AA) content in the hydrogels apparently promoted their surface hydrophilicity as indicated by continuously descending in their water contact angles.

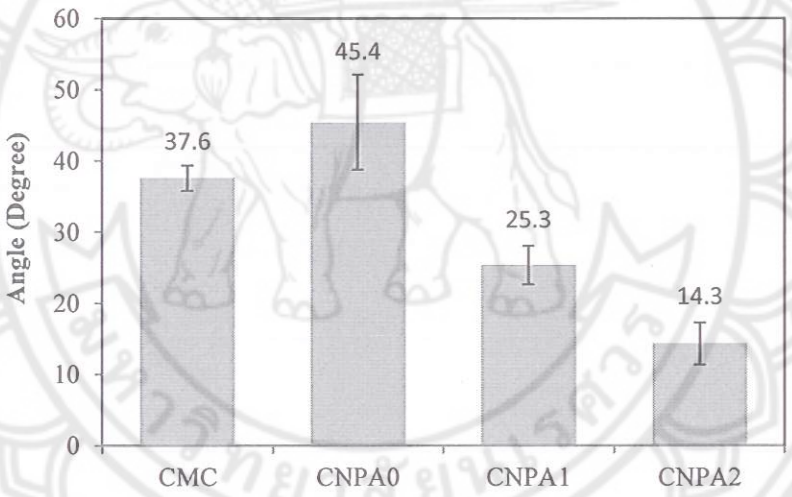


Figure 14 **Water contact angles of CMC, poly(NIPAAm)-grafted CMC hydrogel (CNPA0) and poly(NIPAAm-*co*-AA)-grafted CMC hydrogels (CNPA1 and CNPA2)**

Determination of the phase-transition temperature (LCST) of the hydrogels

Water swelling behavior of the CMC hydrogels modified with poly(NIPAAm) (CNPA0) and poly(NIPAAm-*co*-AA) (CNPA1 and CNPA2) as a function of the solution temperature was investigated (Figure 15). The phase-transition temperature, indicated by the presence of LCST, can be estimated by dividing the EWC *vs* temperature plot into three parts and then drawing the three corresponding tangents. The intersections of the central tangent with the other two tangents were determined (T_1 and T_2) and the center of these two intersections gives rise to the LCST of the sample. An example of the calculation is illustrated in the supporting information. It was observed that as increasing AA concentrations in the hydrogels, %EWC apparently increased, while their LCST values did not significantly change (32°C). The improvement in water swellability of the hydrogels upon addition of poly(AA) was attributed to the presence of highly hydrophilic poly(AA) in the hydrogel structure, which essentially promoted water absorbing capability of the samples. The existence of the phase-transition temperature of these hydrogels were attributed to the presence of thermo-responsive poly(NIPAAm) in their structure. The practically identical LCST values observed in this experiment indicated the formation of block structure of poly(NIPAAm) in the hydrogels.

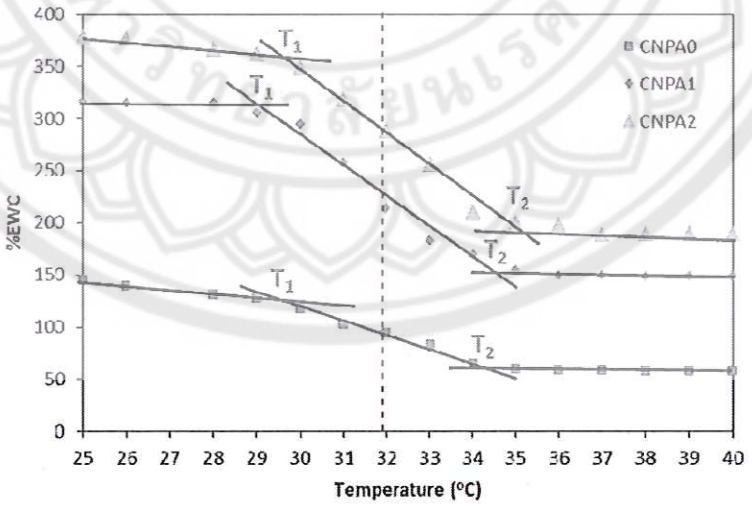


Figure 15 **Temperature dependence of equilibrium water content (EWC) of CNPA0, CNPA1 and CNPA2 hydrogels**

Water swelling studies

1. Water swelling behavior of the hydrogels as a function of temperature

Swelling behaviors of the hydrogels having different polymer compositions in water as a function of solution temperature are shown in the Figure 16. The experiments were performed at three different solution temperatures (10, 30 and 50°C) based on the hypothesis that thermo-responsive poly(NIPAAm) can swell in the solution at the temperature below its LCST and deswell at those above its LCST. In Figure 16a, CMC hydrogel (the control sample) did not show a temperature-dependent behavior due to the absence of thermo-responsive poly(NIPAAm) in its structure. After addition of poly(NIPAAm) in the hydrogels (Figure 16b), it showed a response to the change of its solution temperatures; it collapsed at 50°C and apparently swelled at 10°C in the solution. Amide groups (-NHCO-) in poly(NIPAAm) structure form intermolecular hydrogen bondings with its surrounding water at 10°C, resulting in the swollen state at the temperature below its LCST. On the other hand, at the temperature above its LCST (50°C), poly(NIPAAm) collapsed due to the formation of intramolecular hydrogen bondings.

Grafting poly(AA) into the CMC hydrogels showed an enhancement in their water swelling properties. %EWC significantly increased from 50-130% in the hydrogels without poly(AA) (Figure 16b) to 150-330% in those with poly(AA) (Figure 16c). Increasing poly(AA) concentration in the CMC hydrogels even further promoted their %EWC (180-400%) (Figure 16d). Significant improvement in water swelling properties of these samples was attributed to the presence of carboxylic acid groups in poly(AA) structure. The ionizable carboxylic acid functional groups can promote the formation of hydrogen bondings of the hydrogels to their surrounding water molecules, resulting in the enhancement in water swellability of the hydrogels. It should be noted that the enhancement in hydrophilicity of the poly(AA)-grafted CMC hydrogels observed in the water swelling studies are also in good agreement with those observed in the water contact angle studies.

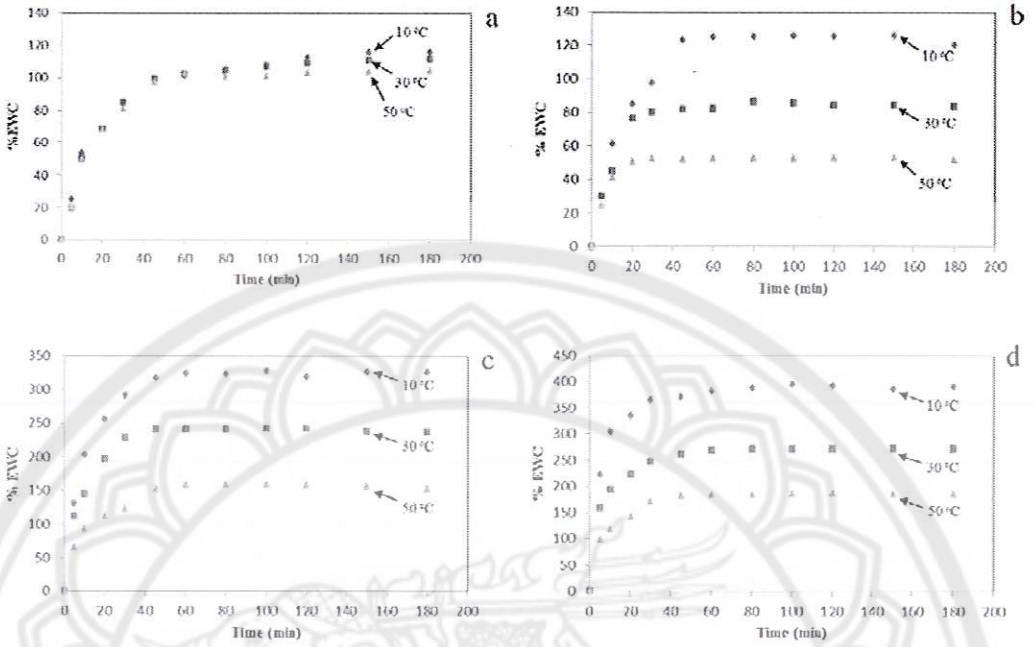


Figure 16 **Equilibrium water content (%EWC) of (a) CMC (b) CNPA0, (c) CNPA1 and (d) CNPA2 hydrogels in aqueous solutions at different temperatures (♦ 10, ■ 30 and ▲ 50 °C)**

2. Water swelling behavior of the hydrogels as a function of pH

The swelling behavior of the hydrogels as a function of solution pH is shown in Figure 17. The experiments were carried at three different solution pHs (4, 7 and 10) to investigate the water swelling behavior of the hydrogels in acidic, neutral and basic pH, respectively. Because the existence of amino and carboxylic acid groups in its structure, CMC hydrogels without poly(NIPAAm) and poly(AA) modification (the control sample) can response to the change of its environmental pH (Figure 17a). The CMC hydrogel exhibited relatively high %EWC in the pH 10 solution as opposed to those at pH 4 and 7, probably due to the formation of carboxylate ions in CMC structure, which essentially promoted hydrogen bondings with water molecules. After addition of poly(NIPAAm) in the hydrogels (Figure 17b), the response to the pH change seemed to be lessened probably due to the presence of non-ionizable poly(NIPAAm) in the hydrogels.

Again, grafting poly(AA) into the CMC hydrogels showed an improvement in their water swellability. For example, in pH 10 solution, %EWC significantly increased from 90% in the hydrogels without poly(AA) (Figure 7b) to 300% in those having poly(AA) (Figure 17c) and even higher to 360% when % poly(AA) in the hydrogel further increased (Figure 17d). The enhancement in water swellability of the hydrogels was again attributed to the formation of carboxylate ions in a basic pH solution, which thus enhanced the formation of hydrogen bondings of the hydrogels to their surrounding water. Interestingly, the response to the change in the solution pH even more obvious when the percentage of poly(AA) in the hydrogels increased (Figure 7c and 7d). This was attributed to the increase in the amount of carboxylic acid groups in the hydrogels, which essentially promoted the sensitivity to the change in its solution pH.

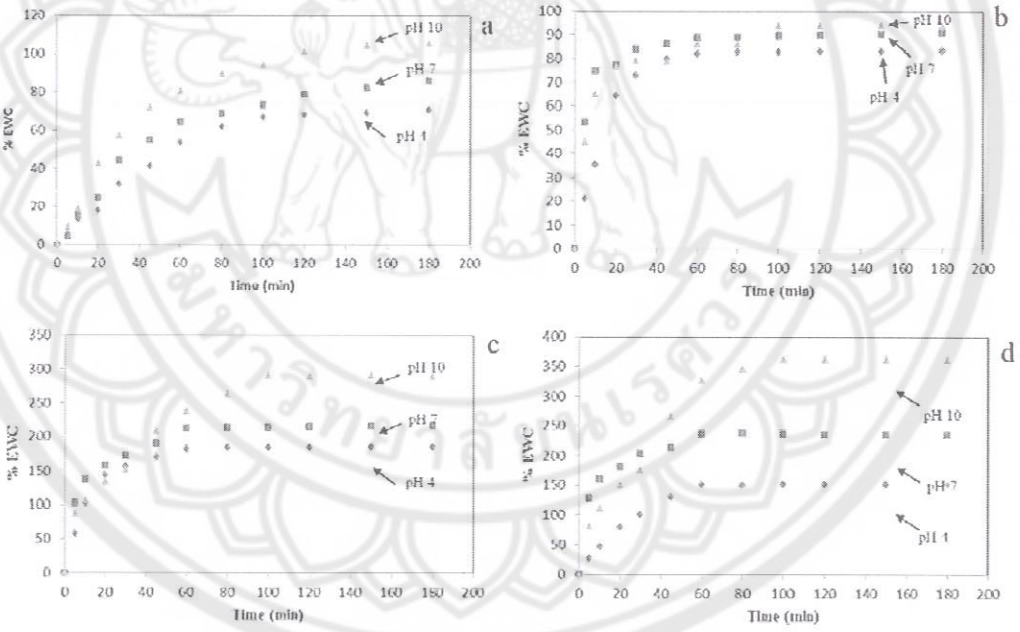


Figure 17 **Equilibrium water content (%EWC) of (a) CMC, (b) CNPA0, (c) CNPA1 and (d) CNPA2 hydrogels in aqueous solution at different pHs (♦ 4, ■ 7 and ▲10)**

Effect of temperature and pH changes on releasing rate of indomethacin

The releasing profiles of indomethacin of CNPA2 were illustrated in Figure 8. The CNPA2 hydrogel was used as a representative of the developed thermo- and pH-responsive hydrogels in the current studies due to the high content of poly(NIPAAm) and poly(AA) in its structure. The concentration of indomethacin in the solution released from the drug-entrapped hydrogels was tracked by UV-visible spectrophotometry. It should be noted that the entrapment and loading efficiencies (%EE and %DLE) of CNPA2 hydrogel were first investigated before studying the drug releasing profiles and it was found that its %EE and %DLE were 18.77% and 1.50%, respectively. An example of the calculation of %EE and %DLE of the hydrogel is illustrated in the supporting information.

The indomethacin releasing profiles of the hydrogel were performed in water as a function of solution temperature (10, 30 and 50°C) and pH (pH 4, 7 and 10) (Figure 8a and 8b, respectively). After 12 h observation, the percentage of the released drug reached the plateau and it was found that high percentage of the drug released at 50oC (80% drug released) was observed as opposed to those at 10oC and 30oC (30% and 65% drug released). It was rationalized that the hydrogel collapsed at the temperature above its LCST (32°C), which essentially accelerated the rejection of the entrapped drug to the aqueous solution.

The drug releasing behavior of the hydrogels was also dependent on the solution pH due to the presence of pH-responsive poly(AA). After 12 h observation, high percent drug release was observed in acidic pH solution (83%) as compared to those in neutral and basic pH solutions (65% and 30%, respectively). This was again attributed to the expelling of the entrapped drug from the collapsed hydrogels in acidic pH solution. It should be noted that these results agree well with the water swelling behavior of the hydrogels showing the collapsed structure in acidic solution and the swollen state in basic solutions.

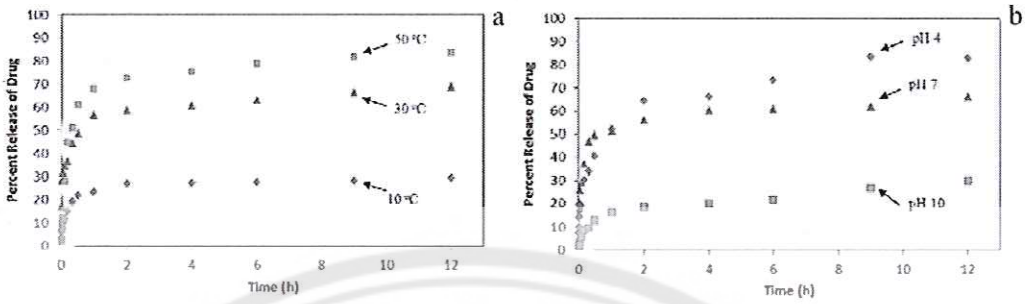


Figure 18 **Releasing behavior of indomethacin of CNPA2 hydrogel in water at different temperatures (♦ 10, ▲30 and ■ 50 °C) and pH (♦ 4, ▲7 and ■ 10)**

Cytotoxicity

Cytotoxicity is an important characteristics of materials intended for use in biomedical applications. In this work, viability of fibroblast cells on the hydrogels was determined by the MTT cytotoxicity assay. It should be noted that if viability of the samples is less than 70% as compared to the blank, they show a cytotoxic potential [ref]. It was found that all samples tested in this experiment, including CMC, CNPA0 and CNPA2 hydrogels, exhibited more than 90% viable cells after 24-hour incubation. This indicated the potential of these hydrogels in biomedical uses.

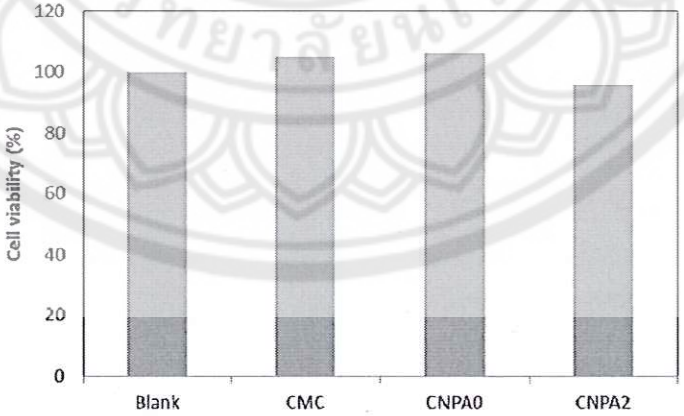


Figure 19 **Cytotoxicity testing of CMC, CNPA0 (the CMC hydrogels grafted with poly(NIPAAm)) and CNPA2 (the CMC hydrogels grafted with poly(NIPAAm-*co*-AA))**

Antibacterial properties

Figure 20 shows the curves of optical density (OD) versus culture time of the tested hydrogels against *S. aureus*, the gram positive bacterial. Because the bacterial cells are opaque, the tested medium will become even more turbid as the bacteria propagate. Therefore, the OD values can be measured during the experiment, which essentially reflect the antibacterial ability of the samples; the less OD of the medium, the higher antibacterial ability of the sample. CMC hydrogels showed antibacterial activity as opposed to the control sample. This was attributed to the presence of amino and carboxylate functional groups in its structure [107-109]. The presence of poly(NIPAAm) in the hydrogels (CNPA0 samples) showed some inhibition in antibacterial activity as compared to the CMC hydrogels. This was probably due to the depletion of the amino and/or carboxylic acid contents in the hydrogel upon grafting poly(NIPAAm) in its structure. On the other hand, the hydrogels grafted with poly(AA) (CNPA2) showed a significant improvement in antibacterial activity. This was again attributed to the increase in the carboxylic acid contents in the hydrogels. These results are in good agreement with the precedents previously reported about the antibacterial ability of the samples containing amino and carboxylic acid functional groups.

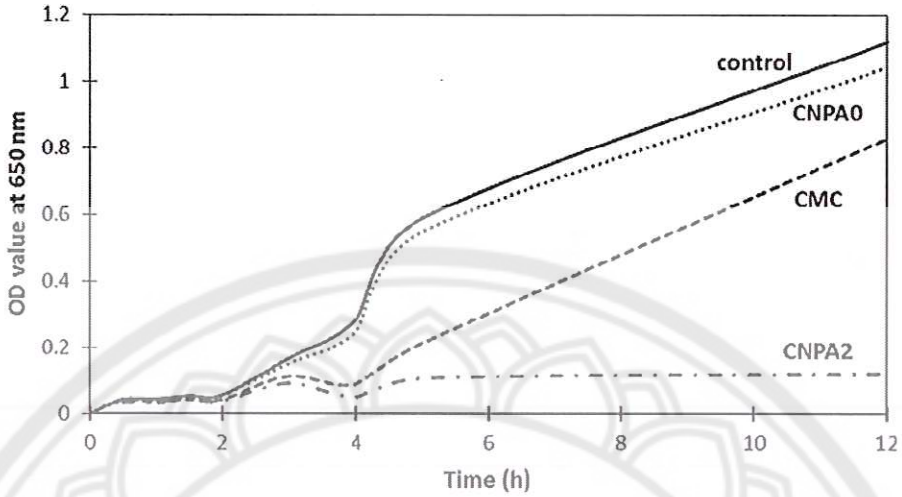


Figure 20 **Evaluation of *in vitro* inhibition ability to *S. aereus* of the hydrogels in 12 h duration.**

CHAPTER V

CONCLUSION

This work illustrated a simple strategy to prepare the "smart" hydrogels based on CMC. The hydrogels containing thermo-responsive poly(NIPAAm) and pH-responsive poly(AA) were synthesized *via* a facile, cost effective and environmentally friendly free radical polymerization in water. The hydrogels showed the responses after exposed to temperature and pH stimuli due to the change in their structures. Due to their non-toxic properties, these novel "smart" hydrogels might be potentially applicable in biomedical uses, such as the hydrogels having dual triggering release mechanisms for drug controlled release applications. In addition, these hydrogels might also be beneficial in the applications requiring antibacterial properties.

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APPENDIX

Appendix A Determination of conversion of NIPAAm monomers *via* ^{1}H NMR spectroscopy

Percent conversion of the poly(NIPAAm) was calculated from the integration ratio of the residual NIPAAm monomer peak to the DMF peak (as an internal standard).

Example;

At 0 min; NIPAAm and DMF have integration area 0.31 and 0.33, respectively.

At 10 min; NIPAAm and DMF have integration area 0.11 and 0.33, respectively.

Thus, % conversion of NIPAAm can be calculated from the following equation:

$$\% \text{ conversion} = \left(1 - \frac{[M]}{[M_O]}\right) \times 100;$$

$[M_0]$ = Integration ratio of poly(NIPAAm)/DMF were used as an initial concentration

$[M_0]$ of Poly(NIPAAm) = 0.31/0.33 = **0.93**

$[M]$ = Concentration of integration ratio of poly(NIPAAm)/ DMF at 10 min reaction

$[M]$ of poly(NIPAAm) = 0.11/0.33 = **0.33**

$$\% \text{ conversion of NIPAAm at 10 min} = \left(1 - \frac{[0.33]}{[0.93]}\right) \times 100$$

$$= \mathbf{64.51\%}$$

Table 2 The conversion of NIPAAm monomers

Time (min)	Integration ratio		NIPAAm/DMF	M_t(NIPAAm)/M_o	%conversion of NIPAAm
	NIPAAm	DMF			
0	**0.31**	**0.33**	**0.93**	**100**	**0**
5	0.16	0.33	0.48	51.61	48.39
10	**0.11**	**0.33**	**0.33**	**35.49**	**64.51**
30	0.08	0.33	0.24	25.81	74.19

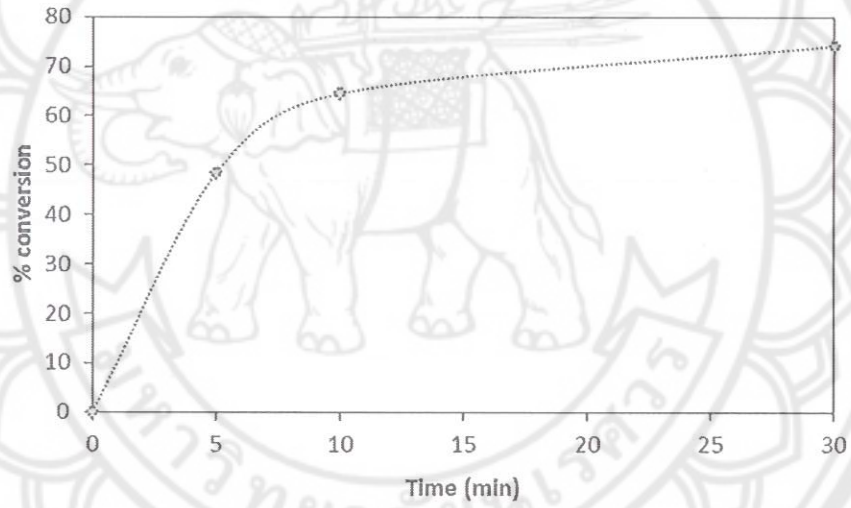

Figure 21 The percent conversion of poly(NIPAAm) as a function of time

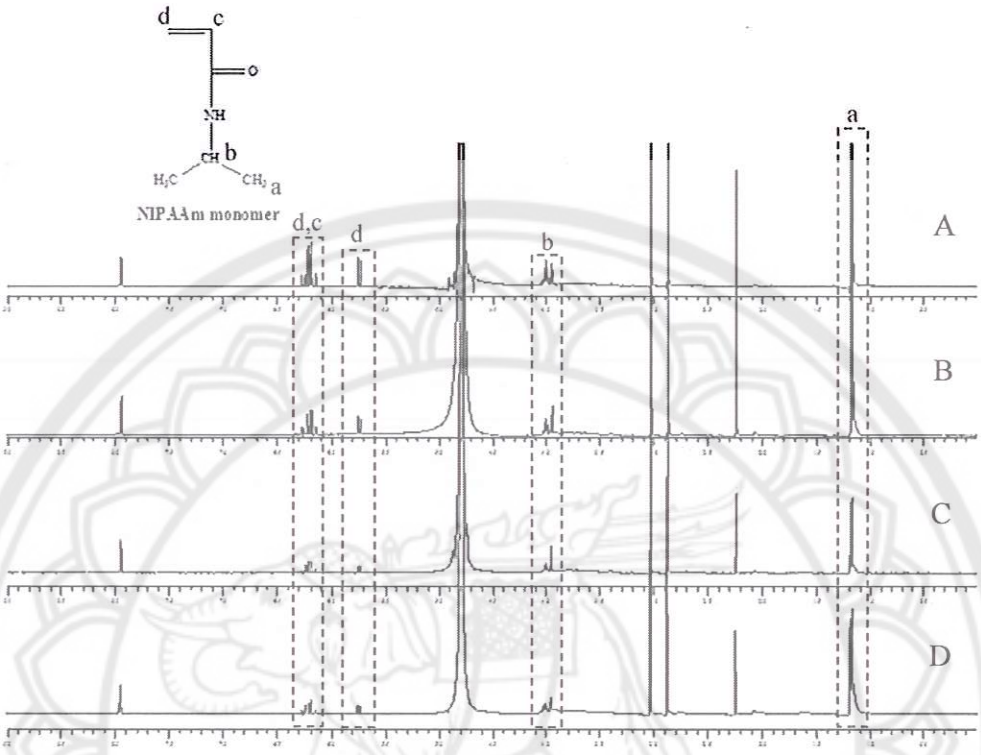

Figure 22 ^{1}H NMR spectra of NIPAAm monomer as calculation their %conversion A) 0 min, B) 5 min), C) 10 min) and D) 30 min

Appendix B The conversion of AA *via* ^{1}H NMR spectroscopy

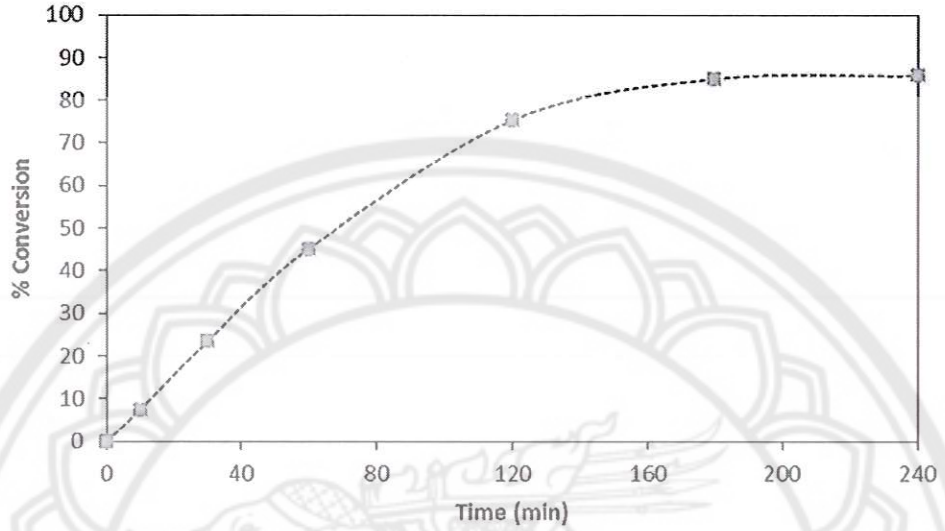

Figure 23 The percent conversion of poly(AA) as a function of time

Table 3 The conversion of AA monomers

Time (min)	Integration ratio		AA/DMF	$M_t(AA)/M_o$	%conversion of AA
	AA	DMF			
0	0.520	0.325	1.599	100.00	0.00
10	0.247	0.167	1.482	92.67	7.33
30	0.398	0.325	1.223	76.50	23.50
60	0.147	0.167	0.880	55.04	44.96
120	0.128	0.325	0.394	24.64	75.36
180	0.040	0.167	0.240	15.01	84.99
240	0.038	0.167	0.228	14.26	85.74

Appendix C The temperature dependence of equilibrium swelling ratio of hydrogels

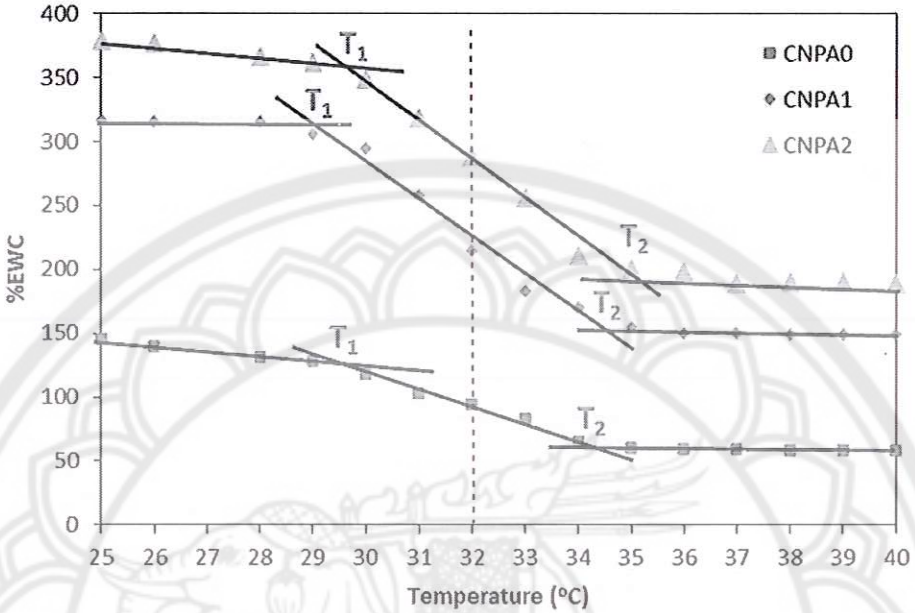

Figure 24 Temperature dependence of equilibrium water content (EWC) of CNPA0, CNPA1 and CNPA2 hydrogels

Table 4 LCST of the CMC hydrogel containing poly(NIPAAm) and poly(AA)

Sample code	Tangent values		LCST
	T_1	T_2	(°C)
CNPA0	29.3	34.4	**31.9**
CNPA1	28.9	34.7	**31.8**
CNPA2	29.8	34.3	**32.1**

Appendix D The studies in water contact angles of the hydrogels

Table 5 Water contact angles *(Θ)* of CMC and the CMC hydrogels grafted with poly(NIPAAm-*co*-AA)

Sample code	Water contact angle (degree)							
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Average	S.D.
CMC	38.2	35.5	37.0	36.6	37.2	40.9	**37.6**	**1.8**
CNPA0	33.0	49.0	52.0	47.6	43.7	47.2	**45.4**	**6.7**
CNPA1	22.0	24.5	25.9	30.0	23.9	25.7	**25.3**	**2.7**
CNPA2	13.3	12.0	11.6	14.3	18.6	16.1	**14.3**	**3.0**

Appendix E An example of the calculation of Grafting Percentage (%G) and Grafting Efficiency (%GE)

Table 6 The weight of CMC and the CMC hydrogels grafted with poly(NIPAAm-*co*-AA)

Sample code	Feed composition of the hydrogel (g)			Dried weight of the hydrogel (g)		
	CMC	NIPAAm	AA	Trail 1	Trial 2	Trial 3
CMC	0.5	-	-	-	-	-
CNPA0	0.5	0.5	-	0.7361	0.7342	0.7355
CNPA1	0.5	0.5	0.08	1.0066	1.0014	0.9951
CNPA2	**0.5**	**0.5**	**0.16**	**1.0967**	1.1006	1.0843

$$\text{Grafting percentage (\%G)} = \frac{W_g - W_c}{W_c} \times 100$$

$$\text{Grafting efficiency (\%GE)} = \frac{W_g - W_c}{W_m} \times 100$$

where W_g= the weights of the dried polymer-grafted CMC

W_c= the weights of the dried CMC

W_m= the weights of the dried monomers (NIPAAm and/or AA)

An example of the calculations of %G and %GE of CNPA2 hydrogel (trail 1)

Grafting percentage (%G) = $\frac{1.0967 - 0.5}{0.5} \times 100$

= **119.34 %**

An example of CNPA2 hydrogel (trail 1)

Grafting efficiency (%GE) = $\frac{10967-08}{08+016} \times 100$

= **90.41 %**

Table 7 Grafting Percentage (%G) of CMC and the CMC hydrogels grafted with poly(NIPAAm-*co*-AA)

Sample code	Grafting percentage (%G)				
	Trail 1	Trail 2	Trial 3	Average	S.D.
CMC	-	-	-	-	-
CNPA0	47.22	46.84	47.10	47.05	0.19
CNPA1	101.32	100.28	99.02	100.21	1.15
CNPA2	**119.34**	120.12	116.86	118.77	1.70

Table 8 Grafting Efficiency (%GE) of CMC and the CMC hydrogels grafted with poly(NIPAAm-*co*-AA)

Sample code	Grafting efficiency (%GE)				
	Trail 1	Trail 2	Trial 3	Average	S.D.
CMC	-	-	-	-	-
CNPA0	47.22	46.84	47.10	47.05	0.19
CNPA1	87.34	86.45	85.36	86.39	0.99
CNPA2	**90.41**	91.00	88.53	89.98	1.29

Appendix F An example of the calculation of percent crosslinking

Table 9 Percent crosslinking of CMC and the CMC hydrogels grafted with poly(NIPAAm-*co*-AA) before and after dissolution

Sample code	Dried weight before dissolution, W_1 (g)			Dried weight after dissolution, W_2 (g)		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
CMC	0.0157	0.0161	0.0161	0.0117	0.0121	0.0122
CNPA0	0.0251	0.0288	0.0304	0.0210	0.0243	0.0252
CNPA1	0.0329	0.0344	0.0352	0.0297	0.0308	0.0315
CNPA2	**0.0369**	0.0384	0.0385	**0.0350**	0.0363	0.0369

$$\text{Percent crosslinking}\,(\%) = \frac{W_2}{W_1} \times 100$$

where W_1 = the weights of the dried sample before dissolutions

W_2 = the weights of the dried sample after dissolutions

An example of the calculation of %crosslinking of CNPA2 hydrogel (Trial 1)

$$\text{Percent crosslinking}\,(\%) = \frac{0.0350}{0.0369} x100$$

$$= \mathbf{94.85\ \%}$$

Table 10 Percent crosslinking (%) of CMC and the CMC hydrogels grafted with poly(NIPAAm-*co*-AA)

Sample code	Percent crosslinking (%)				
	Trial 1	Trial 2	Trial 3	Average	S.D.
CMC	74.52	75.16	75.78	75.15	0.63
CNPA0	83.67	84.38	82.89	83.75	0.74
CNPA1	90.26	89.39	89.47	89.47	0.48
CNPA2	**94.85**	94.53	95.84	95.08	0.64

Appendix G Equilibrium water content (%EWC) of CMC hydrogels containing poly(NIPAAm) and poly(AA) having CMC:NIPAAm:AA at 1:2:2 molar ratio

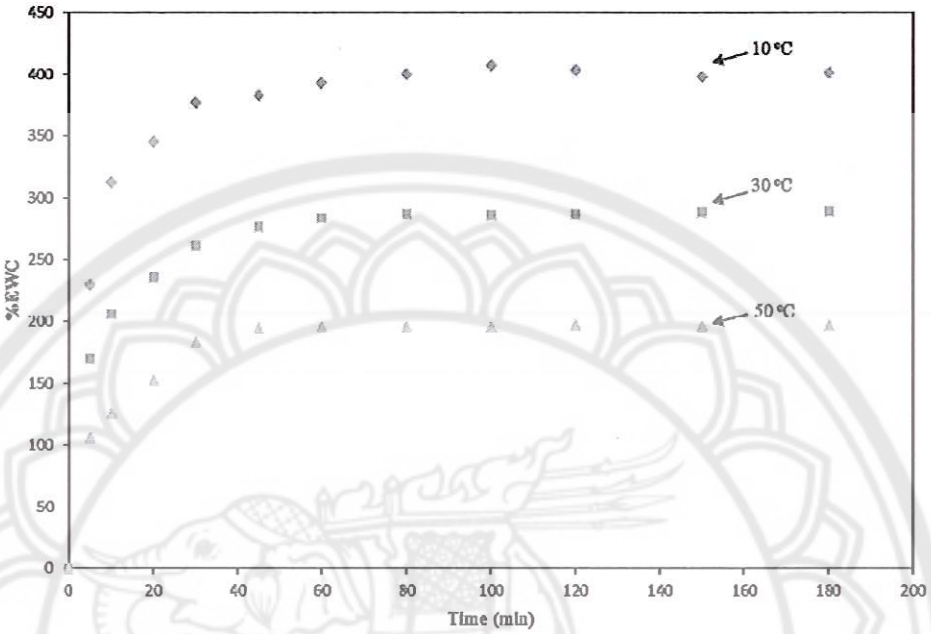

Figure 25. Equilibrium water content (%EWC) of CMC hydrogels at different aqueous solution temperatures (♦ 10, ■ 30 and ▲ 50 °C)

Appendix H Calculation of indomethacin entrapment efficiency (%EE)

Table 11 Percent entrapment efficiency (%EE) determined *via* UV-visible spectrophotometry

Type of hydrogel used	Wt of loaded drug (mg)	Wt of the entrapped drug in complex (mg)	% EE
CNPA2	64	12.12	18.94

Entrapment efficiency (%EE) was determined from the following equation:

$$\%\text{Entrapment Efficency (\%EE)} = \frac{\text{Weight of the entrapped drug in the complex}}{\text{Weight of loaded drug}} \cdot 100$$

Calculation of the weight of the loaded indomethacin

The loaded indomethacin solution was prepared by 65 mg of indomethacin dissolves in 10 ml ethanol and was 200-time diluted with ethanol. After that, the weight of the loaded indomethacin was determined *via* calibration curve of standard indomethacin curve. The observed concentration of indomethacin from UV technique was 32 ppm

Therefore, the weight of indomethacin in the solution $= \frac{(32\ mg)(10\ ml)(200)}{1000\ ml}$

$= 64$ mg

Calculation of the residue of drug in the solution after loading indomethacin

The weight of the entrapped drug in the complex was determined from the difference of the weights of the loaded drug and the drug remaining dispersible in the solution.

The observed concentration of indomethacin residue from UV technique was 25.94 ppm

10 ml of indomethacin-loaded hydrogel was 200-time diluted with EtOH.

Therefore, the weight of indomethacin residue in the solution

$$= \frac{(25.94\ mg)(10\ ml)(200)}{1000\ ml}$$

$$= 51.88 \text{ mg}$$

Calculation of the entrapped drug in the complex

The entrapped drug in the complex = the weight of the loaded drug - the excess of the drug in the solution

The entrapped drug in the complex = (64 mg) – (51.88 mg)

$$= \mathbf{12.12} \text{ mg}$$

Therefore, $\%EE = \frac{12.12\,\text{mg}}{64\ mg} \times 100 = 18.94\ \%w/w$

Appendix I: Calculation of drug (indomethacin) loading efficiency (%DLE)

Table 12 Percent drug (indomethacin) loading efficiency (%DLE) determined *via* UV-visible spectrophotometry

Type of hydrogel used	Wt of hydrogel (mg)	Wt of the entrapped drug in complex (mg)	% DLE
CNPA2	8.002×10^2	12.12	1.52

Drug (indomethacin) loading efficiency (%DLE) was determined from the following equation:

$$\%\text{Drug Loading Efficiency (\%DLE)} = \frac{\text{Weight of entrapped drug in the complex}}{\text{Weight of nanoparticles}} \cdot 100$$

The weight of the hydrogel = $\mathbf{8.002 \times 10^2}$ mg

Calculation of the entrapped indomethacin was illustrated in the above example of %EE.

Thus,

$$\%\text{DLE} = \frac{(12.12\ mg)(100)}{800.2\ \text{mg}} = \mathbf{1.52}\ \%\text{w/w}$$

Appendix J Cytotoxicity testing

Table 13 The average percent viability of mouse fibroblast…..of the samples

Sample	The average of	
	OD 570 nm	%Viability
Blank	**1.504**	100
Negative control	1.479	98
Positive control	0.015	1
CMC	1.581	105
CNPA0	1.597	106
CNPA2	**1.450**	**96**

If viability is reduced to <70% of the blank, it has a cytotoxic potential.

Remark: Viab% = $100 \times OD_{570c}/OD_{570b}$

OD_{570c} = the mean value of the measured optical density of the test sample

OD_{570b} = the mean value of the measured optical density of the blanks

In the case of CNPA2 hydrogel

Viab% = (1.450/1.505) x 100

= **96 %**

Appendix K Antibacterial activity

Table 14 The absorbance of the solution samples as a function of time

Time	The absorbance of sample (OD)			
(h)	Control	CMC	CNPA0	CNPA2
0	0	0	0	0
0.5	0.042	0.044	0.039	0.038
1	0.044	0.036	0.041	0.039
1.5	0.054	0.045	0.05	0.045
2	0.056	0.044	0.055	0.041
3	0.167	0.114	0.152	0.093
4	0.283	0.09	0.247	0.052
5	0.588	0.213	0.547	0.108
12	1.118	0.829	1.041	0.123

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Material behaviour

"Smart" carboxymethylchitosan hydrogels crosslinked with poly(*N*-isopropylacrylamide) and poly(acrylic acid) for controlled drug release

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ABSTRACT

Carboxymethylchitosan (CMC) hydrogels containing thermo-responsive poly(*N*-isopropylacrylamide) (poly(NIPAAm)) and pH-responsive poly(acrylic acid) (poly(AA)) were prepared *via* a free radical polymerization in the presence of hexamethylene-1,6-di-(aminocarboxysulfonate) crosslinking agents. A proper ratio of CMC to NIPAAm and AA used in the reaction was investigated such that the thermo- and pH-responsive properties of the hydrogels were obtained. Water swelling of the hydrogels was improved when the solution pH was in basic conditions (pH 10) or the temperature was below its lower critical solution temperature (LCST). Effects of the change in solution temperature and pH on water swelling properties of the hydrogel as well as the releasing rate of an entrapped drug were also investigated. The hydrogels were not toxic and showed antibacterial activity against *Straphylococcus aureus* (*S. aureus*). The pH- and thermo-responsive properties of this novel "smart" hydrogel might be efficiently used as dual triggering mechanisms in controlled drug release applications.

1. Introduction

Recently, hydrogels have been widely used in many potential application areas such as medicine [1], biotechnology [2], industry [3] and environmental science [4]. A hydrogel is a network of hydrophilic polymers that can swell and absorb a large amount of aqueous solution while maintaining its structure [5]. Crosslinking of the polymer chains in hydrogels can form three-dimensional networks. Different mechanisms can be used to form the crosslinks such as, covalent bonding, hydrogen bonding, van der Walls interaction or physical entanglements [6–8]. Interestingly, hydrogels can also control drug-releasing behavior by changing the gel structure in responses to changes in its environment. A hydrogel containing such "sensor" properties can undergo reversible volume phase transition with only minute changes in the environmental conditions and is considered as a "smart" or "intelligent" hydrogel [9–11].

Also during recent years, carboxymethylchitosan (CMC), a natural amphoteric polyelectrolyte [12], has attracted considerable interest in a wide range of biomedical applications, such as wound dressings, artificial bone and skin, and bacteriostatic agents due to its good biocompatibility

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and low toxicity [13,14]. CMC is a chitosan derivative having carboxymethyl substituents on some amino and/or primary hydroxyl sites of the glucosamine units of chitosan. It shows good pH and ion sensitivity in aqueous solutions due to abundant ionizable -COOH and $-NH_2$ functional groups in its structure. CMC is readily soluble in water [15,16], while CMC hydrogel (a crosslinked form) swells significantly in basic solutions and shrinks in acidic solutions. CMC hydrogel has thus been widely studied for use in controllable drug delivery.

Generally, pH-responsive polymers are polyelectrolytes [17,18] that bear weak acidic or basic groups in their structure [19]. These allow them to either protonate or deprotonate in response to the change in their environmental pH. Typical examples of this class of polymers are poly(carboxylic acids), in particular poly(acrylic acid) (poly(AA)). It swells in basic pH solutions due to the formation of carboxylate anions and collapses in acidic pH solutions [20,21] because the carboxylic groups are protonated and unionized.

Another class of "smart" polymer that is now of great interest is polymers that possess thermo-responsive properties. They have been increasingly investigated for use in several biomedical applications [22] such as selective bio-separation [23,24], smart bioactive surfaces [25,26] and phase separation immune-assays [27,28]. These polymers usually have a lower critical solution temperature (LCST), which is the characteristic phase transition temperature of each polymer. Below the LCST, the enthalpy term relating to the hydrogen bonding between polar groups of the polymer and water molecules dominates, leading to dissolution of the polymer in water. Above the LCST, the entropy term relating to hydrophobic interactions among the polymers dominates [29], resulting in precipitation of the polymer in water. Poly(*N*-isopropylacrylamide) (poly(NIPAAm)) is the best-known thermo-responsive polymer in this class because it exhibits a LCST at 32 °C [30,31] which is somewhat close to that of the human body (37 °C) in aqueous solution [32–34]. Therefore, poly(NIPAAm), a thermo-responsive polymer [35], has been used widely in the fields of chemistry, material science and biotechnology [36].

The aim of this work was to develop a novel thermo- and pH-responsive hydrogel based on CMC with dual triggering mechanisms for controlled drug release. This hydrogel is comprised of biocompatible CMC, thermo-responsive poly(NIPAAm) and pH-responsive poly(AA) using a water soluble crosslinking agent (hexamethylene-1,6-di-(aminocarboxysulfonate) or HDA) to form a semi-interpenetrating polymer network (semi-IPN) (Fig. 1). Water contact angle measurement was carried out to study surface properties of the hydrogel and scanning electron microscopy (SEM) was performed to investigate its morphology. Swelling characteristics of the hydrogel as a function of solution temperature and pH were investigated. The release profiles of indomethacin, a model drug, from the hydrogels were studied. Additionally, cytotoxicity and antibacterial activity of the hydrogel were investigated.

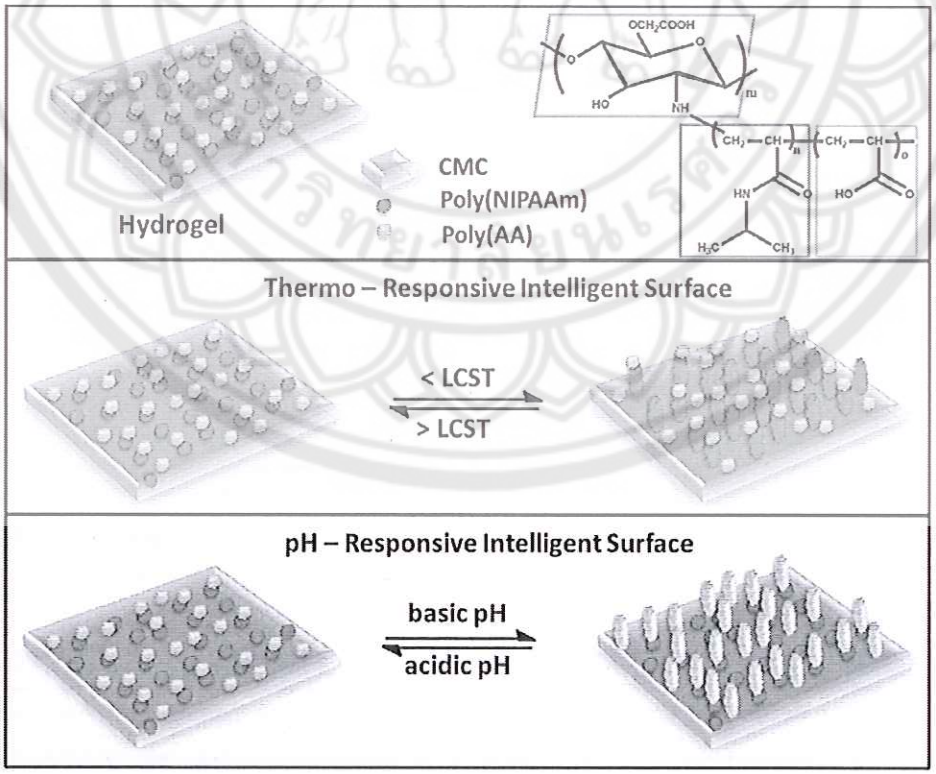

Fig. 1. (A) Modification of CMC hydrogels with thermo-responsive poly(NIPAAm) and pH-responsive poly(AA), (B) and (C) ideal swelling/deswelling behavior upon changing its environmental temperature and pH, respectively.

2. Experimental

2.1. Materials

Chitosan from crab ($\overline{M}_n$ = 1.4 × 10^5 g/mol) (Taming Enterprise, 98% deacetylation) was used without purification. *N*-isopropylacrylamide (NIPAAm) (Acros, 99%) was recrystallized in hexane before use to remove inhibitors. Acrylic acid (AA) (Acros, 99.5%) was distilled under reduced pressure before use. 1,6-Hexamethylene diisocyanate (HDI)(Carlo Erba, 99%), diammonium peroxodisulphate (APS) (Carlo Erba, 98%), sodium metabisulfate ($Na_2S_2O_4$) (Carlo Erba, 97%) and indomethacin (Sigma, 90%) were used as received. All other chemicals were analytical-grade and used without further purification.

2.2. Syntheses

2.2.1. Synthesis of carboxymethylchitosan (CMC) from chitosan

Chitosan (40 g) swollen in isopropanol (100 ml) for 24 h was reacted with a NaOH solution at room temperature for 75 min, and then reacted with monochloroacetic acid (40 g, 0.51 mol in H_2O 100 ml) at 60 °C for 5 h. The solution was then precipitated in an excess of methanol. To remove salts, it was washed with a methanol:H_2O solution (70:30 v/v). CMC was filtered and dried at 40 °C for 24 h.

2.2.2. Synthesis of hexamethylene-1,6-di-(aminocarboxysulfonate) (HDA) as a water soluble crosslinker

1,6-Hexamethylene diisocyanate (HDI) (6.73 g, 20 mmol) was introduced into a sodium metabisulfate solution (8.36 g, 40 mmol in 15 ml of H_2O) and the mixture was then stirred at room temperature for 24 h. The product was precipitated in an excess of acetone, which was then filtered and dried *in vacuo*.

2.2.3. Synthesis of poly(NIPAAm-co-AA) -grafted with CMC hydrogels

An example for the synthesis of CNPA2 is illustrated in Table 1. Other CMC hydrogels were prepared in a similar fashion with proper amounts of reagents used. CMC (0.5 g, 2.3 mmol of carboxymethyl glucosamine unit) and NIPAAm (0.5 g, 4.4 mmol) were dissolved in DI water (10 ml) with stirring under N_2 for 30 min at room temperature. After heating to 60 °C, APS (0.0025 g 8.9 mmol), a radical initiator was added to the solution, which was then stirred for 45 min. AA (0.16 g, 2.2 mol) was then added as a co-monomer into the solution with stirring for 4 h. HDA (0.05 g, 0.1 mmol), a crosslinking agent, was introduced into the solution with continuously stirring for another 30 min. After the reaction was completed, the solution was dried *in vacuo* at 40 °C for 24 h to form the hydrogel. To remove ungrafted poly(NIPAAm) and poly(AA), the hydrogel was immersed in excess acetone for 24 h, filtered and dried. It should be noted that ungrafted poly(NIPAAm) and poly(AA) are soluble in acetone but the covalently crosslinked poly(NIPAAm-*co*-AA) in CMC hydrogel is insoluble.

To synthesize the hydrogel, poly(NIPAAm) was first covalently grafted onto CMC chains *via* a free radical chain polymerization using APS as an initiator. Amine radicals (·NH-) can be formed on CMC chains, which give rise to the initiating site for poly(NIPAAm)-grafted CMC. The reaction was allowed for 30 min to reach 30% conversion of NIPAAm as determined *via* ^{1}H NMR spectroscopy. In the case of CNPA0, the reaction was ceased at this low percent conversion to avoid a premature chain termination due to radical recombination. In the cases of CNPA1 and CNPA2 (Table 1), appropriate amounts of AA monomers were sequentially added to the reaction vessels to further extend the polymer chains from the grafted poly(NIPAAm), leading to the formation of poly(NIPAAm-*co*-AA)-grafted CMC (Fig. 1). After 4 h of poly(AA) polymerization, ^{1}H NMR spectroscopy indicated 80% conversion of AA.

It should be mentioned that free poly(NIPAAm) and poly(AA) homopolymers and copolymers might also be formed due to the existence of ammonium sulfate radicals in the solution, which act as free radical initiators in formation of the hydrogels. These polymers were left in the solution without extraction in order to simplify the hydrogel preparation process. It is envisioned however, that the ungrafted poly(NIPAAm) and poly(AA) that might exist in the solution are physically locked in the CMC hydrogels *via* chain entanglement (without covalent bonds). These free polymers are thus embedded in poly(-NIPAAm-*co*-AA)-grafted CMC using a water-soluble HDA crosslinker to form semi-IPN.

2.3. Characterization of the polymers and hydrogels

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) was recorded on a Bruker NMR spectrometer operating at 400 MHz. Fourier transformed infrared spectroscopy (FTIR) was conducted on a Perkin-Elmer Model 1600 series FTIR spectrophotometer using KBr pellets. Morphological studies of the sample surface were carried out through LEO 1455 VP scanning electron microscopy (SEM) with an accelerating voltage of 5 kV. To prepare the hydrogel for SEM experiments, it was swollen in water at 10 °C for 24 h and then lyophilized. The dried film was cut into 1 × 1 cm^2 pieces, adhered to aluminum stubs and coated with gold. Grafting percentage (%G) and efficiency (%GE) were estimated by the difference in the weights before and after the grafting reactions, calculated according to the following equations:

$$\text{Grafting percentage (\%G)} = (W_g - W_c)/W_c \times 100\% \quad (1)$$

Table 1
Feed composition for the preparation of copolymer hydrogels.

Sample	CMC (g)	NIPAAm (g)	AA (g)	molar ratio of CMGA[a]: NIPAAm:AA	%G[b]	%GE[c]
CMC	0.5	–	–	–	–	–
CNPA0	0.5	0.5	–	1:2:0	47.05 ± 0.19	47.05 ± 0.19
CNPA1	0.5	0.5	0.08	1:2:0.5	100.21 ± 1.15	86.39 ± 0.99
CNPA2	0.5	0.5	0.16	1:2:1	118.77 ± 1.70	89.98 ± 1.29

[a] CMGA is carboxymethylglucosamine unit in CMC.
[b] %G is grafting percentage.
[c] %GE is grafting efficiency.

$$\text{Grafting efficiency (\%GE)} = (W_g - W_c)/W_m \times 100\% \quad (2)$$

where W_g, W_c and W_m are the weights of dried polymer-grafted CMC, CMC and monomers (NIPAAm and/or AA), respectively.

2.4. Water contact angle measurement

Contact angles (θ) between water and sample films were investigated using the sessile method on a Ramé-Hart Model 200 Standard Contact Angle Goniometer at room temperature. A drop of water was carefully applied on a sample film and the contact angle was quickly measured before it started to swell. The reported values are the average of five different measurements.

2.5. Determination of percent crosslinking

The dried films with the dimension of 1 × 1 cm^2 were immersed into DI water and stirred at room temperature for 24 h to dissolve uncrosslinked portions in the hydrogel. The insoluble hydrogel was filtered and thoroughly washed with distilled water and acetone to further remove untrapped portions. The swollen gels were then dried at 30 °C for 24 h. Percent crosslinking was calculated as following:

$$\text{Percent crosslinkling} = \frac{W_2}{W_1} \times 100 \quad (3)$$

where W_1 and W_2 are the weights of dried samples before and after dissolutions, respectively. The reported values are the average of at least three different measurements.

2.6. Determination of water swelling behavior

Equilibrium water content (%EWC) of the hydrogels was investigated by immersing the dried films in an aqueous solution at a given temperature and pH. The swollen films were periodically removed from the solution and excess water on their surface was wiped off. %EWC was calculated from the following equation;

$$\text{EWC}(\%) = \frac{W_s - W_d}{W_d} \times 100 \quad (4)$$

where W_d and W_s are the weights of dried and swollen samples, respectively.

2.7. Determination of entrapment efficiencies (%EE) and drug loading efficiencies (%DLE)

The dried hydrogels were submerged in an indomethacin-ethanol solution (0.065 mg of indomethacin in 10 ml of ethanol) at 10 °C for 2 days to fully swell the drug into the hydrogels. The difference of the weights of indomethacin in the solutions before and after the swelling experiments was determined by UV-visible spectrophotometry at a wavelength of 320 nm, and this result reflected the weight of the entrapped drug in the hydrogel. Therefore, %EE and %DLE were calculated using the following equations;

$$\text{\% Entrapment efficiency (\%EE)} = \frac{\text{Weight of the entrapped drug in the hydrogel}}{\text{Weight of the loaded drug}} \times 100 \quad (5)$$

$$\text{\% Drug loading efficiency (\%DLE)} = \frac{\text{Weight of the entrapped drug in the hydrogel}}{\text{Weight of the dried film}} \times 100 \quad (6)$$

2.8. Studies in the in vitro drug release behavior

Indomethacin release behavior of the drug-entrapped hydrogels was determined as a function of solution temperature and pH. To study the effect of the solution pH on drug release behavior, 1 × 1 cm^2 pieces of dried films were immersed in a phosphate buffer solution (PBS) at 25 °C at pH 4, 7 or 10. Similar experiments were performed in PBS at pH 7.4 at 10, 30 or 50 °C to study the effect of solution temperature on drug release. The drug concentration in the releasing media was periodically determined *via* UV-vis spectrophotometry (320 nm).

2.9. Cytotoxicity

The cytotoxicity test was carried out using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) cytotoxicity assay. Cell culture experiments were carried out using mouse fibroblasts. Cell suspension of 1 × 10^5 cells/ml L929 in Minimum Essential Medium (MEM) was seeded in a 96-well plate and incubated at 37 ± 1 °C with 5.0 ± 0.1% CO_2 and 95 ± 5% relative humidity for 24 ± 2 h to obtain confluent monolayers of cells prior to use. The dried hydrogels were sterilized in an autoclave at 121 °C for 15 min. A 'Thermanox' (Nunc) coverslip and a polyurethane film containing 0.1% Zinc diethyldithiocarbamate (ZDEC) were used as negative and positive control materials, respectively. After incubation, the viable cells were stained with MTT and incubated for 2 h. Then, MTT was removed and dimethylsulfoxide (DMSO) was added in each well. The absorbance was measured using a microplate reader at 570 nm.

2.10. Antibacterial activity

Antibacterial activity of the hydrogels against *S. aureus* (*S. aureus*, TISTR 1466) was evaluated using the optical density (OD) method, measured by a shake flask test. Briefly, the dried hydrogel (0.1 g) was submerged in Mueller Hinton broth (HiMedia Laboratories Pvt. Ltd., India) medium (20 ml) containing 10^6 CFU/mL *S. aureus* and then the medium was incubated at 37 °C with shaking (150 rpm). The bacterial suspension without the hydrogel was set as a control. During the incubation process, the turbidity of the medium was measured at 650 nm every hour for 12 h. The bacterial growth was reported in terms of the OD value. Each measurement was performed under aseptic conditions using aseptic techniques.

3. Results and discussion

The main objective of this study was to prepare dual response CMC-based hydrogels that have thermo-responsivity poly(NIPAAm) and pH-responsivity poly(AA). Poly(NIPAAm) and poly(AA) were embedded into highly water-swollen CMC using a water soluble crosslinking agent (hexamethylene-1,6-di-(aminocarboxysulfonate) or HDA) to form a semi-interpenetrating polymer network (semi-IPN). The dual-responsive properties of CMC hydrogel were then used as triggering mechanisms for controlled release of indomethacin model drug.

Grafting percentage (%G) of the polymers in the CMC chains were in the range of 47-119 %, while their grafting efficiencies (%GE) ranged between 47% and 90% (Table 1). These numbers increased significantly when poly(AA) present in the hydrogels (CNPA1 and CNPA2). This was attributed to the extension of the chain lengths of the grafted polymers on CMC when the reaction time was prolonged. Increasing AA incorporated in the reactions (CNPA2) also promoted both %G and %GE of the hydrogels.

3.1. Functional group characterization of the hydrogels

The functional groups of the as-synthesized poly(-NIPAAm-*co*-AA)-grafted CMC hydrogels were analyzed *via* FTIR (Fig. 2). Poly(NIPAAm) and poly(AA) homopolymers were prepared in a separate aliquots for analyses of the characteristic signals of their functional groups. As compared to the spectra of the homopolymers (Fig. 2b and c) and CMC (Fig. 2a), the copolymers exhibited the characteristic absorption signals of amide functional groups of poly(NIPAAm): 1650 cm^{-1} (NH-CO- stretching), 1548 cm^{-1} (N-H bending) and 3436 cm^{-1} (N-H stretching), and the signals of carboxylic acid groups of poly(AA): 1736 cm^{-1} (HO-CO- stretching) and 3436 cm^{-1} (O-H stretching).

3.2. Determination of percent crosslinking of the hydrogels

HDA was used as a water soluble crosslinking agent for the formation of CMC hydrogels. Percent crosslinking of poly(NIPAAm-*co*-AA)- grafted CMC hydrogels was investigated in comparison with those of CMC hydrogels without addition of the polymers (Fig. 3). It was found that addition of poly(NIPAAm) and/or poly(AA) into CMC seemed to increase percent crosslinking of the hydrogels, which was attributed to the increase of network density due to the increase of polymer content in the structure.

3.3. Surface morphology studies

Fig. 4 illustrates the morphology studies of fully water-swollen hydrogels after lyophilization. SEM images of the CMC hydrogels without poly(AA) (CMC and CNPA0) (Fig. 4A

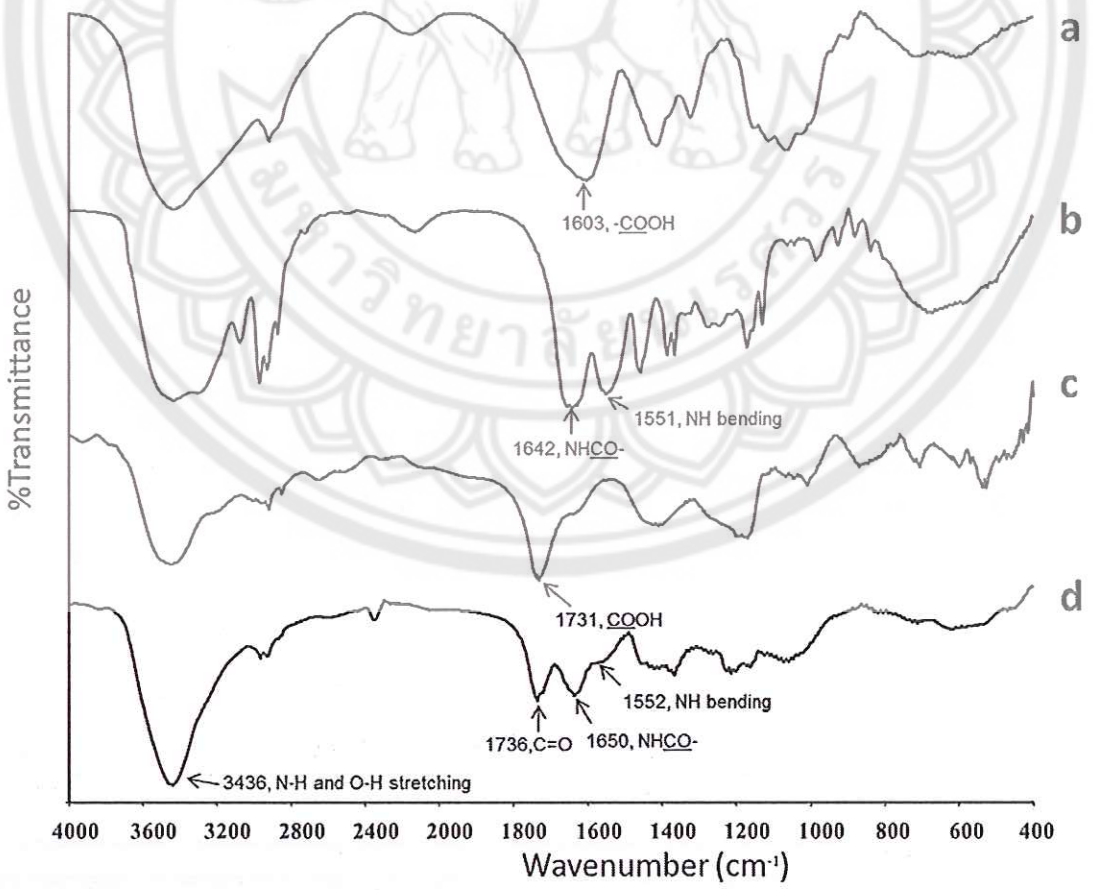

Fig. 2. FTIR spectra of (a) CMC, (b) poly(NIPAAm), (c) poly(AA) and (d) poly(NIPAAm-*co*-AA)-grafted CMC hydrogel.

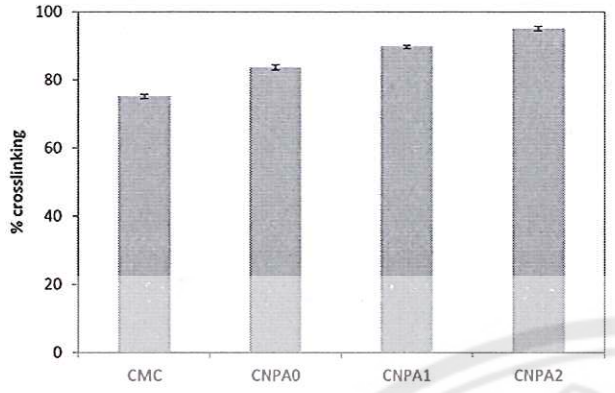

Fig. 3. Percent crosslinking of CMC hydrogels and poly(NIPAAm-*co*-AA)-grafted CMC hydrogels.

and B) showed dense morphologies, while those of CNPA1 and CNPA2 exhibited abundant open and porous structure (Fig. 4C and D). These micropores existed on their surface and also in the bulk of the hydrogels (Fig. 4C-F). It was rationalized that hydrophilic poly(AA) might promote water uptake and swelling, leading to the formation of micropores in the swollen hydrogels. In addition, when the hydrophilic poly(AA) contents in the hydrogels were increased from 2:0.5 to 2:1.0 molar ratios of NIPAAm:AA, respectively (CNPA1 and CNPA2 in Table 1), the degree of porosity of the hydrogels even further increased as evidenced in Fig. 4E and F. In good agreement with the SEM results, the CMC grafted with poly(AA) showed an increase in water absorption as opposed to those without poly(AA) and this will be discussed in detail in the water swelling study section.

3.4. Water contact angle studies

Effect of poly(NIPAAm) and poly(AA) grafted on CMC on surface wettability of the hydrogels was investigated by measuring their water contact angles in comparison to the CMC hydrogels without the polymers grafting. Hypothetically, increasing water contact angle implies a decrease in surface hydrophilicity of the material. It was found that the increase in water contact angle from 37.6° to 45.4° was attributed to the presence of hydrophobic isopropyl groups of poly(NIPAAm), resulting in the enhancement of surface hydrophobicity of the hydrogels (Fig. 5). Increasing amounts of hydrophilic poly(AA) in the hydrogels (CNPA0, CNPA1 and CNPA2) apparently enhanced their surface hydrophilicity, as indicated by continuously decrease of their water contact angles. These results suggest that poly(NIPAAm) and poly(AA) in CMC hydrogels might be able to migrate to the hydrogel-air interface while crosslinking.

3.5. Determination of LCST of the hydrogels

Water swelling behavior of the CMC hydrogels modified with poly(NIPAAm) (CNPA0) and poly(NIPAAm-*co*-AA) (CNPA1 and CNPA2) as a function of the solution temperature was investigated (Fig. 6). The phase-transition temperature, is indicated by the presence of a LCST that can be estimated by dividing the %EWC *vs* temperature plot into three parts and then drawing the three corresponding tangents. The intersections of the central tangent with the other two tangents were determined (T_1 and T_2), and the center of these two intersections indicates the LCST of the sample. With increasing amount of AA in the hydrogels, the %EWC increased, while their LCST values did not change (32 °C). The improvement in water swellability of the hydrogels when adding poly(AA) was owing to the presence of highly hydrophilic poly(AA) in the hydrogel structure, which enhanced water swellability of the samples. The existence of the phase-transition temperature of these hydrogels was attributed to the presence of thermo-responsive poly(NIPAAm) in their structure, and this was confirmed by the unchanged %EWC of CMC hydrogels without poly(NIPAAm) at the same temperature range (25-40 °C).

3.6. Water swelling properties

3.6.1. Temperature dependence of water swelling properties of the hydrogels

Water swelling behaviors of the hydrogels as a function of temperature are shown in the Fig. 7. The experiments were performed at three different solution temperatures (10, 30 and 50 °C) based on the hypothesis that thermo-responsive poly(NIPAAm) swells in water at a temperature below its LCST and shrinks at temperatures above its LCST. In Fig. 7a, CMC hydrogels (the control sample) did not show temperature-dependent behavior because there was no thermo-responsive poly(NIPAAm) in its structure. After addition of poly(NIPAAm), the hydrogels (Fig. 7b) showed a response to the change of its solution temperature; it collapsed at 50 °C and apparently swelled at 10 °C in the solution. Amide groups (-NHCO-) in poly(NIPAAm) structure form intermolecular hydrogen bonding with its surrounding water at 10 °C, resulting in the swollen state below its LCST. On the other hand, at a temperature above its LCST (50 °C), poly(NIPAAm) collapsed due to the formation of intramolecular hydrogen bonding.

Grafting hydrophilic poly(AA) into CMC hydrogels showed enhancement of their water swelling properties. % EWC significantly increased from 50-130% in the hydrogels without poly(AA) (Fig. 7b) to 150-330% in those with poly(AA) (Fig. 7c). Increasing poly(AA) concentration in the CMC hydrogels even further promoted their %EWC (180-400%) (Fig. 7d). Significant improvement in water swelling properties of these samples was attributed to the presence of carboxylate groups in the poly(AA) structure. Carboxylate groups can promote the interaction of the hydrogels with surrounding water molecules, resulting in the enhancement of water swellability of the hydrogels. It should be noted that the enhancement of hydrophilicity of the poly(AA)-grafted CMC hydrogels observed in the water swelling studies are also in good agreement with those observed in the water contact angle studies.

3.6.2. pH dependence of water swelling properties of the hydrogels

The swelling behavior of the hydrogels as a function of pH is shown in Fig. 8. The experiments were performed

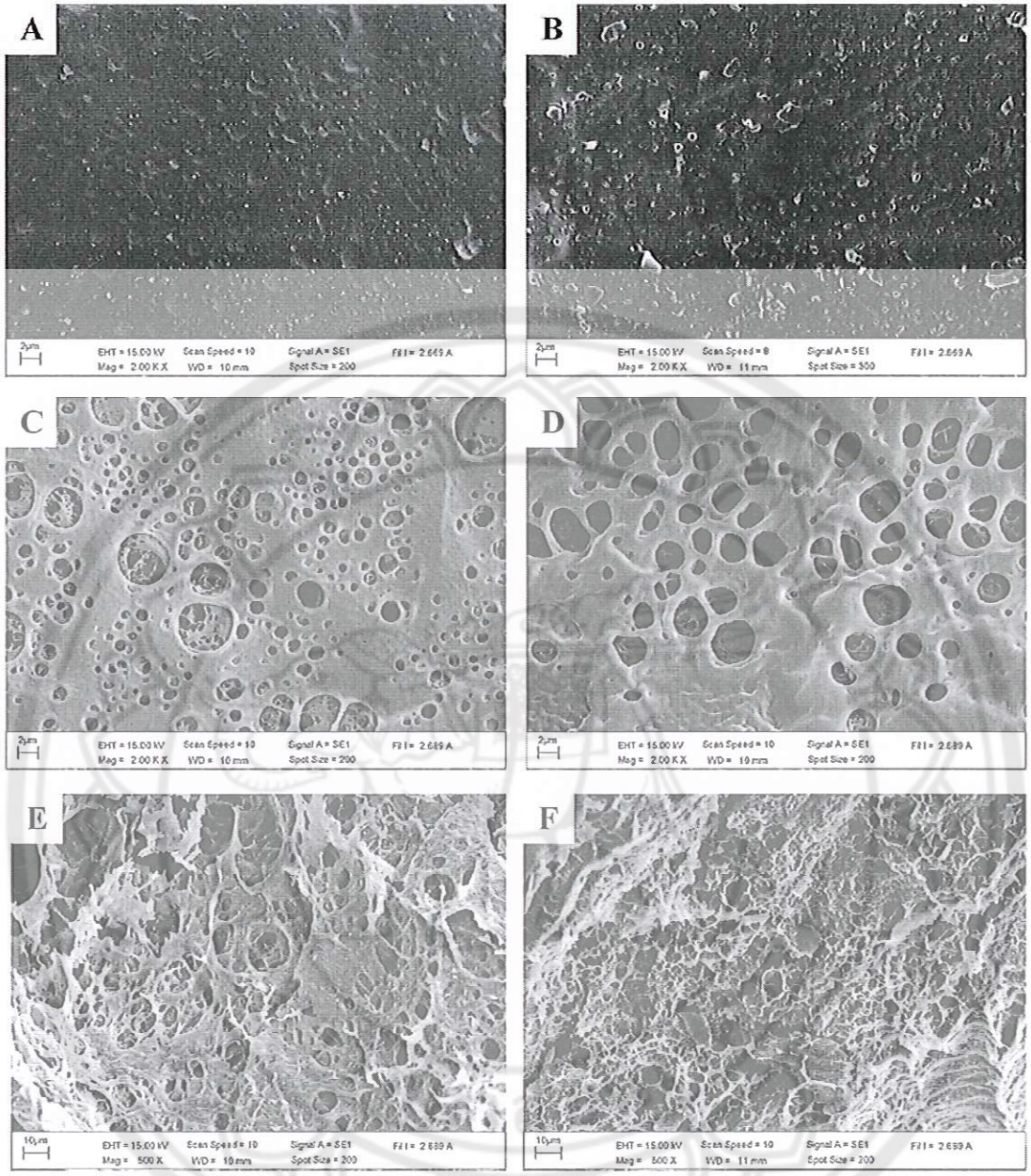

Fig. 4. Surface morphology of (A) CMC, (B) CNPA0 (C) CNPA1, (D) CNPA2 hydrogels, and cross-sectional morphology of (E) CNPA1 and (F) CNPA2 hydrogels.

in water with acidic, neutral and basic pHs (pH 4, pH 7 and pH 10). Because of the existence of amino and carboxylic acid groups in CMC chains, CMC hydrogels without poly(NIPAAm) and poly(AA) modifications (the control samples) showed responses to change of the solution pH (Fig. 8a). The CMC hydrogel exhibited relatively high %EWC in the pH 10 solution as opposed to those at pH 4 and 7, probably due to the formation of carboxylate ions in CMC structure, which essentially promoted the interactions with water molecules. After addition of poly(NIPAAm) (Fig. 8b), the response to the pH change seemed to be lessened, probably due to the presence of non-ionizable poly(NIPAAm) in the hydrogels.

Again, grafting poly(AA) into CMC, hydrogels showed an improvement in their water swellability. For example, in

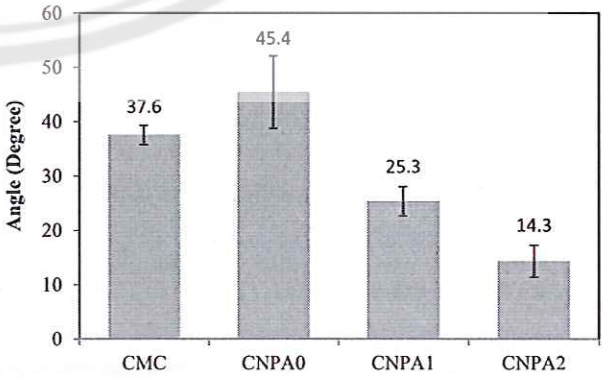

Fig. 5. Water contact angles of CMC, CNPA1 and CNPA2 hydrogels.

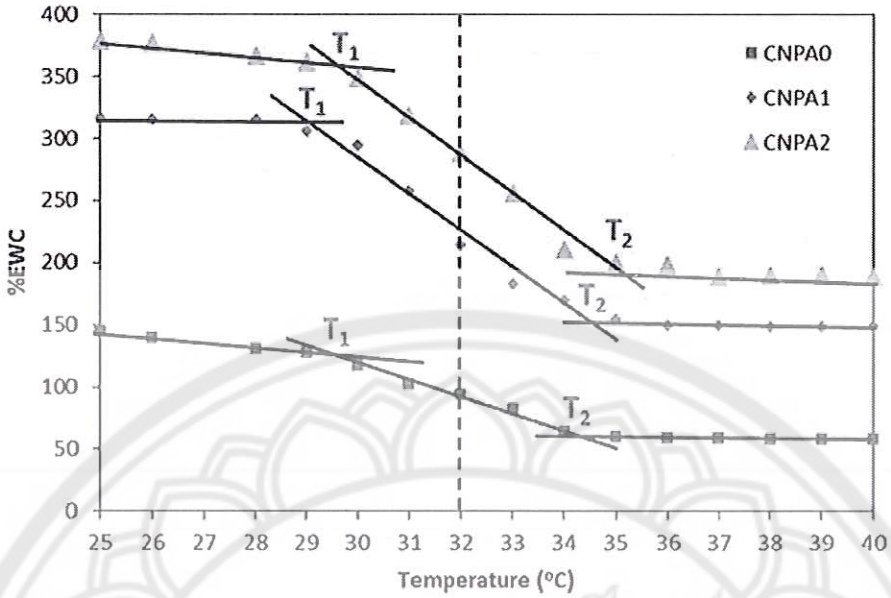

Fig. 6. Temperature dependence of equilibrium water content (EWC) of CNPA0, CNPA1 and CNPA2 hydrogels.

pH 10 solutions, %EWC significantly increased from 90% in the hydrogels without poly(AA) (Fig. 8b) to 300% in those having poly(AA) (Fig. 8c) and even higher to 360% when % poly(AA) in the hydrogels further increased (Fig. 8d). The enhancement in water swellability of the hydrogels was again attributed to the formation of carboxylate ions in a basic pH solution, which thus enhanced the interactions of the hydrogels with water. Interestingly, the response to the change in the solution pH even more obvious when the percentage of poly(AA) in the hydrogels increased (Fig. 8c

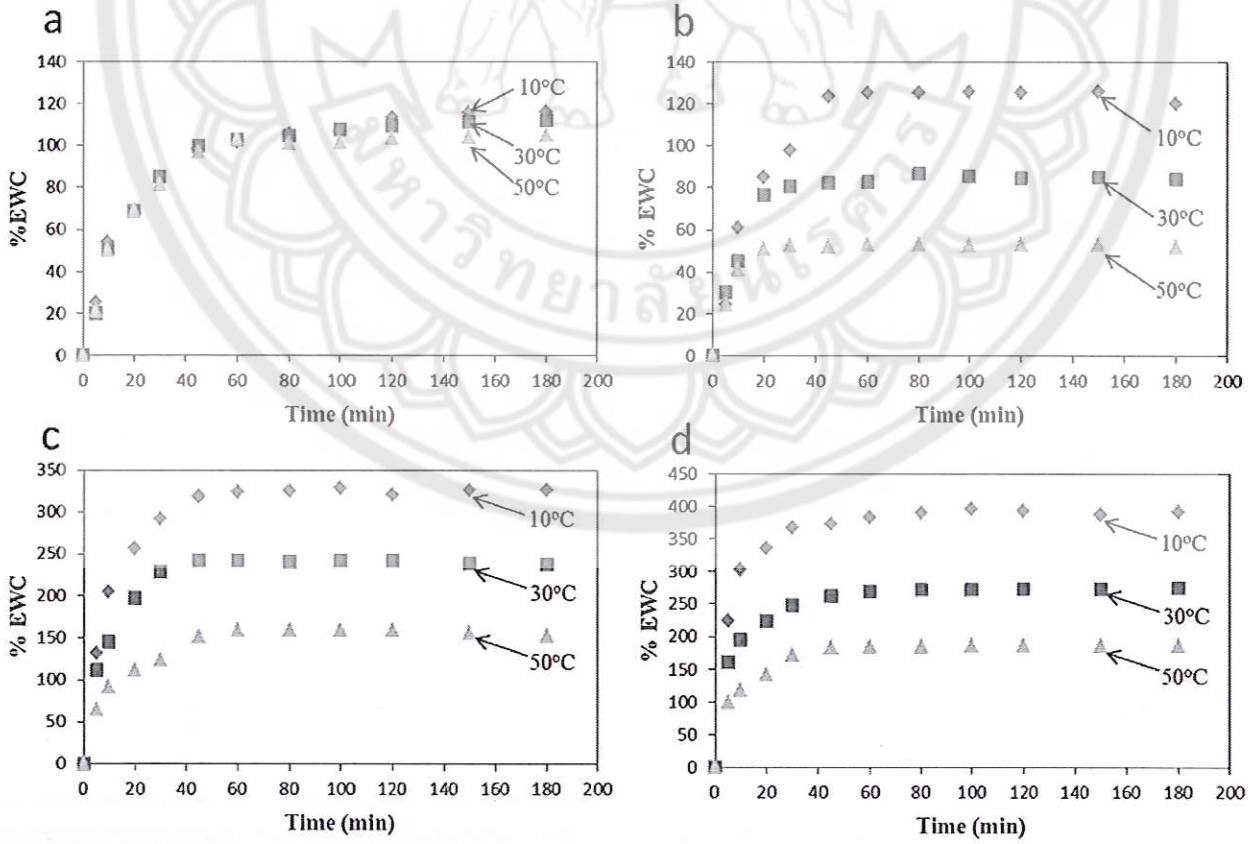

Fig. 7. %EWC of (a) CMC (b) CNPA0, (c) CNPA1 and (d) CNPA2 hydrogels in water at different temperatures (◆10, ■ 30 and ▲50 °C).

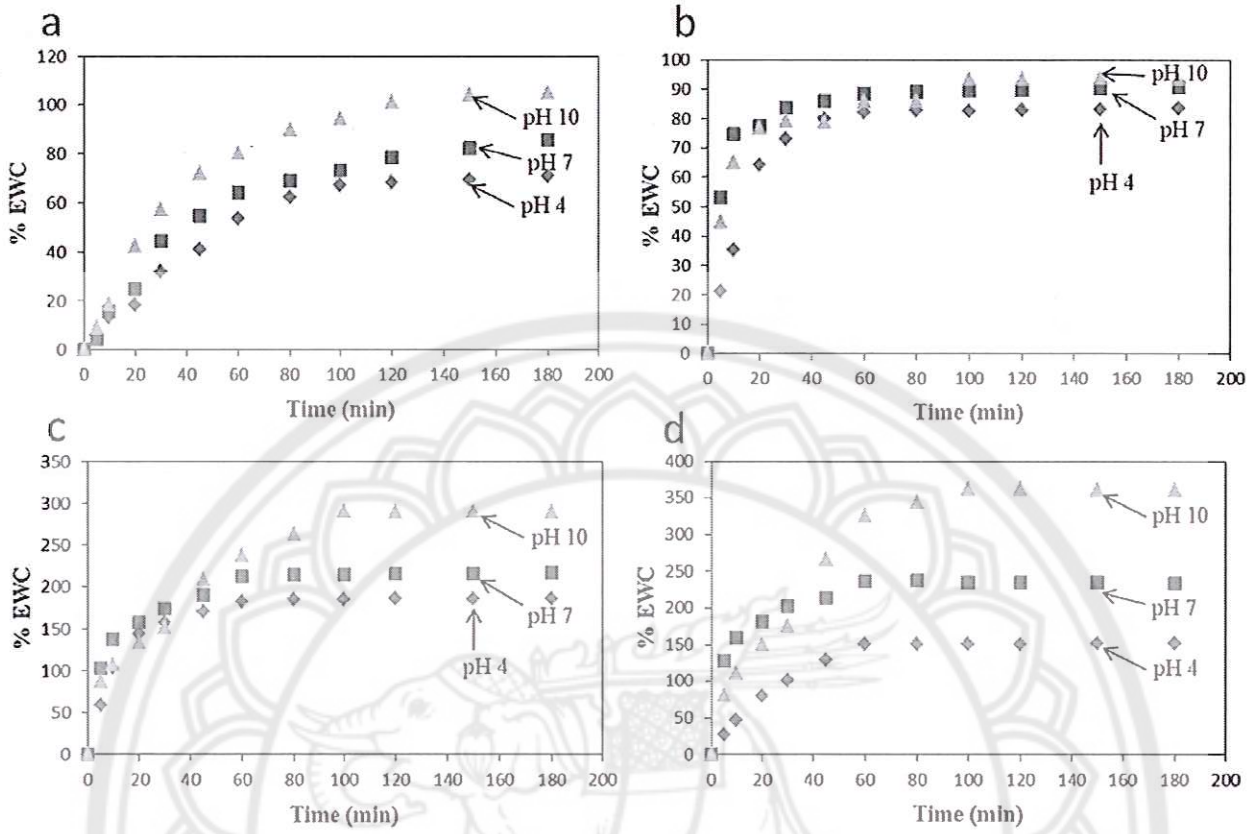

Fig. 8. %EWC of (a) CMC, (b) CNPA0, (c) CNPA1 and (d) CNPA2 hydrogels in water at different pHs (◆ pH 4, ■ pH 7 and ▲ pH 10).

and d). This was attributed to the increase in the amount of carboxylate groups in the hydrogels, which essentially promoted the sensitivity to the change in its solution pH.

3.7. *Effect of temperature and pH changes on releasing rate of indomethacin*

As CNPA2 hydrogels had relatively high water swellability and showed good responses to the changes of both pH and temperature, they were selected as representative samples for controlled drug release studies. The release profiles of indomethacin of CNPA2 are illustrated in Fig. 9. The concentration of indomethacin in the solution released from the drug-entrapped hydrogels was tracked by UV-visible spectrophotometry. It should be noted that the entrapment and loading efficiencies (%EE and %DLE) of CNPA2 hydrogels were first investigated before

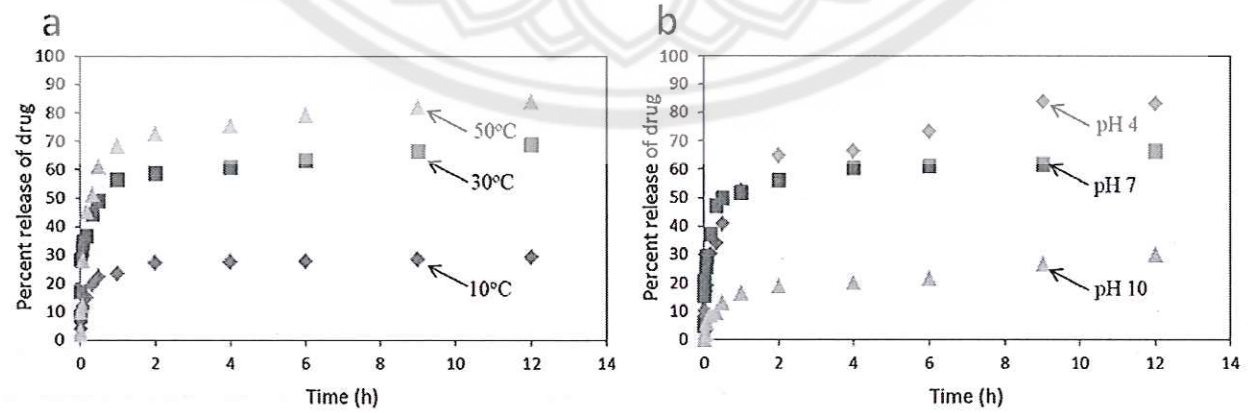

Fig. 9. Releasing behavior of indomethacin of CNPA2 hydrogels in water at different temperatures (◆10, ■ 30 and ▲50 °C) and pH (◆ pH 4, ■ pH 7 and ▲ pH 10).

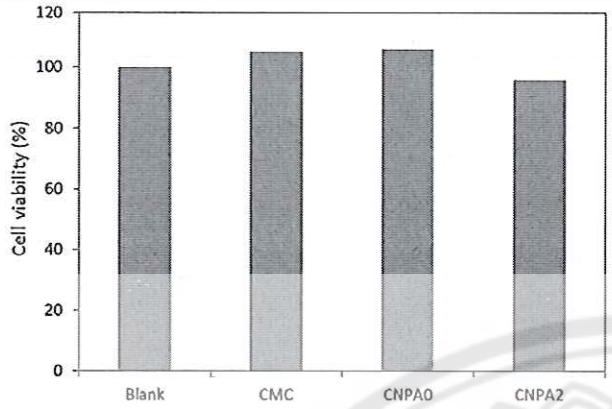

Fig. 10. Cytotoxicity testing of CMC, CNPA0 (poly(NIPAAm)-grafted CMC hydrogels) and CNPA2 (poly(NIPAAm-*co*-AA)-grafted CMC hydrogels).

studying the drug releasing profiles, and it was found that its %EE and %DLE were 18.77% and 1.50%, respectively.

The indomethacin release profiles of the hydrogels were obtained in water as a function of solution temperature (10, 30 and 50 °C) and pH (pH 4, pH 7 and pH 10) (Fig. 9a and b, respectively). After 12 h observation, the percentage of the released drug reached a plateau and it was found that a high percentage of the drug was released at 50 °C (80% drug released) as opposed to 30% and 65% at 10 °C and 30 °C, respectively. It was rationalized that the hydrogel collapsed at a temperature above its LCST (32 °C), which essentially accelerated the rejection of the entrapped drug to the aqueous solution.

The drug releasing behavior of the hydrogels was also dependent on the solution pH due to the presence of pH-responsive poly(AA). After 12 h observation, a high percent drug release was observed in acidic pH solution (83%) as compared to in neutral and basic pH solutions (65% and 30%, respectively). This was again attributed to the expelling of the entrapped drug from the collapsed hydrogels in acidic pH solution. It should be noted that these results agree well with the water swelling behavior of the hydrogels, showing the collapsed structure in acidic solution and the swollen state in basic solutions.

3.8. Cytotoxicity

Cytotoxicity is an important characteristics of materials intended for use in biomedical applications. In this work, viability of fibroblast cells on the hydrogels was determined by MTT cytotoxicity assay. It should be noted that, if viability of the samples is less than 70% as compared to the blank, they show a cytotoxic potential. It was found that all samples tested in this experiment, including CMC, CNPA0 and CNPA2 hydrogels, exhibited more than 90% viable cells after 24-hour incubation (Fig. 10). This indicated the potential of these hydrogels in biomedical uses.

3.9. Antibacterial properties

Fig. 11 shows the growth curves of *S. aureus* with and without the tested hydrogels. Because the bacterial cells are opaque, the tested medium will become more turbid as the number of bacteria increase. Therefore, the OD values measured during the experiment reflect the antibacterial ability of the samples; the less OD of the medium, the higher antibacterial ability of the sample. CMC hydrogels showed antibacterial activity as opposed to the control sample. This was attributed to the presence of amino and carboxylate functional groups in its structure [37–39]. The presence of poly(NIPAAm) in the hydrogels (CNPA0 samples) showed some inhibition in antibacterial activity as compared to the CMC hydrogels. This was probably due to the depletion of the amino and/or carboxylic acid contents in the hydrogel upon grafting poly(NIPAAm) in its structure. On the other hand, the hydrogels grafted with poly(AA) (CNPA2) showed a significant improvement in antibacterial activity. This was again attributed to the increase in the carboxylic acid contents in the hydrogels. These results are in good agreement with the precedents previously reported about the antibacterial ability of the samples containing amino and carboxylic acid functional groups [37–39].

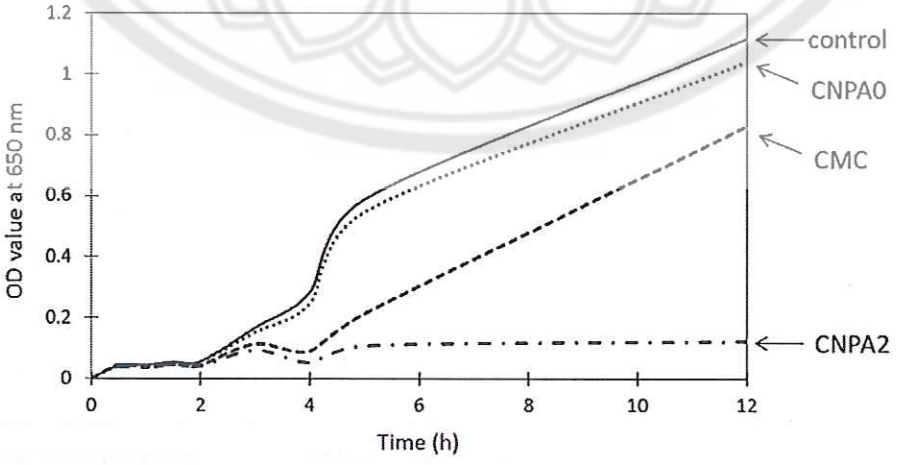

Fig. 11. Antibacterial activity of CMC, CNPA0 and CNPA2 hydrogels against *S. aereus*.

4. Conclusions

This work illustrated a simple strategy to prepare "smart" hydrogels based on CMC. The hydrogels containing thermo-responsive poly(NIPAAm) and pH-responsive poly(AA) were synthesized *via* a simple, cost effective and environmentally friendly free radical polymerization in water. The hydrogels showed responses after exposure to temperature and pH stimuli due to the change in their structures. Due to their non-toxic properties, these novel "smart" hydrogels might be potentially applicable in biomedical uses, such as the hydrogels having dual triggering release mechanisms for controlled drug release applications. In addition, these hydrogels might also be beneficial in the applications requiring antibacterial properties.

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