CHAPTER 1

Introduction

1.1 Historical Background

The burden of cancer is seriously increasing and leading to cause of death worldwide. The population aging and growth as well as a stressful lifestyle, eating habit, smoking and living in a harmful environment with pollution, radiations and pesticides are the causes of cancer [1,2]. Cancer not only affects the lives and working ability of people, but also affects their family, public health and economy. It should be concerned to have researches for solving, preventing, or reducing these problems, otherwise it would seriously impact to the global public health.

Cancer can be treated by chemotherapy, radiation therapy and surgery. The conventional cancer therapeutic agents often restrict by dose limiting toxicity and exhibit poor targeting to tumor region. They also have severe side effects and high costs. Thus, the investigation of new substances for anticancer has been attractive especially for natural antioxidants produced in medicinal plants such as quercetin [3,4], resveratol [5,6], catechins [7,8], carotenoids [9,10] and curcuminoids [11,12] which become intensively investigation for their prevention and treatment of cancer. Natural antioxidants do not expose serious side effects on normal cells.

Curcuminis a promising natural antioxidant which can be found in many plants especially in turmeric. It has been interested in pharmacological researches for recent years. Curcumin demonstrated various health benefits ranging from antioxidant [13-15], anti–inflammatory [16,17] and anticancer properties [4,18,19]. It can inhibit free radicals from mediating lipid peroxidation of membranes or oxidative DNA damage which are the important initiator for carcinogenesis [20,21]. Moreover, it can inhibit the proliferation of many types of cancer cells by binding to many signaling pathways. Clinically, it is considered extremely safe while administered at very high doses [22]. In

spite of such high activities, the pharmaceutical role has been restrictive because of its extremely low aqueous solubility, insufficient tissue absorption and degradation at alkaline pH which hampers its use as therapeutic agent [23-25].

Many technologies have been developed and applied to overcome the major limitation of curcumin. Nanotechnology is one of interesting approaches that has the desirable advantages of improving aqueous solubility of hydrophobic compounds to render them suitable for pharmaceutical formulations, such as liposomes, microemulsions, and polymeric micelles. Using liposomes have some disadvantages which are inconsistent entrapment of drug [26]. Unfortunately, most of liposomes can be destroyed by bile salts and phospholipase enzyme [27]. Although microemulsions are easy to prepare and stable [28] but the high amount of surfactants can induce the tissue irritation after administration [26]. Polymeric micelles have been recently recognized as an important and attractive class of drug carriers. They have shown great promises in solubilization and (targeted) delivery of hydrophobic drugs for chemotherapy [29-32]. They also protect the active compound from the possible destroying under the biological environment [33]. Furthermore, the usage of an appropriate polymer has gained many advantages because of its biocompatibility, biodegradability, narrow size distribution, safety, and reproducibility [34]. Importantly, pharmaceutical production of polymeric micelles has been proven and some formulations have entered clinical evaluations [35,36].

The key to success in this study is to develop curcumin loaded polymeric micelles in order to enhance solubility and increase stability of curcumin. Besides, the antioxidant capacity was performed in comparison with other antioxidants. The kinetic degradation of curcumin under various conditions (pH, temperature, and dielectric constant of the suitable medium) was investigated as the method for determining degradation kinetics of curcumin providing the degradation profile as a basis in comparison of its free form and polymeric micellar formulations. The novel polymers with aromatic ending group, ω–methoxy poly(ethylene glycol)–b–(*N*–(2–benzoyloxy methacrylamide) (PEG–HPMA–Bz), was synthesized and investigated for solubility and stability enhancement and as nanocarrier systems of curcumin compared with the

other two derivatives of methoxypoly(ethylene glycol)–*N*–(2–hydroxypropyl) methacrylamide (PEG–HPMA) based block copolymers modified with monolactate, dilactate, and benzoyl side groups. The physicochemical properties, releasing behavior, degradation of curcumin were evaluated in each carrier system. Also, the *in vitro* pharmacological activities of curcumin–loaded HPMA–based polymeric micelles were performed to evaluate the cytocompatibility on normal cells and cytotoxicity against various types of cancer cell lines. Moreover, the biological activities of curcumin in HPMA–based polymeric micelles were examined on cell cycle arrest and Wilms' tumor 1 (WT1) protein expression to confirm curcumin activity after loading into HPMA–based polymeric micelles in order to get insight into the use of these formulations for cancer therapy.

1.2 Objectives

1.2.1 To synthesize the novel copolymer used as polymeric micelles for curcumin

1.2.2 To enhance the solubility of curcumin using polymeric micelles

1.2.3 To enhance the stability of curcumin by polymeric micelles

1.2.4 To examine the physicochemical characteristics of curcumin–loaded polymeric micelles

1.2.5 To examine the potential of curcumin–loaded polymeric micelles on biological activity

1.3 Literature Review

1.3.1 Curcumin

Curcumin ([1,7–bis(4–hydroxy–3–methoxyphenyl)–1,6–heptadiene–3,5–dione]) is a natural yellow colored phenolic antioxidant. Curcumin was first extracted in an impure form by Vogel *et al*. [37]. The structure of curcumin was elucidated and it was synthesized by Lampe and Milobedzka[38]. Many different plant species synthesize curcumin and the commercial product of curcumin (such as from Sigma–Aldrich) is isolated from the rhizome of *Curcuma longa* L. in which it presents in relatively high concentrations. The chemical structures of curcuminoids are shown in Figure 1.1. It should be mentioned that the commercially available curcumin products also contain three structurally related compounds (i.e., 77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin).

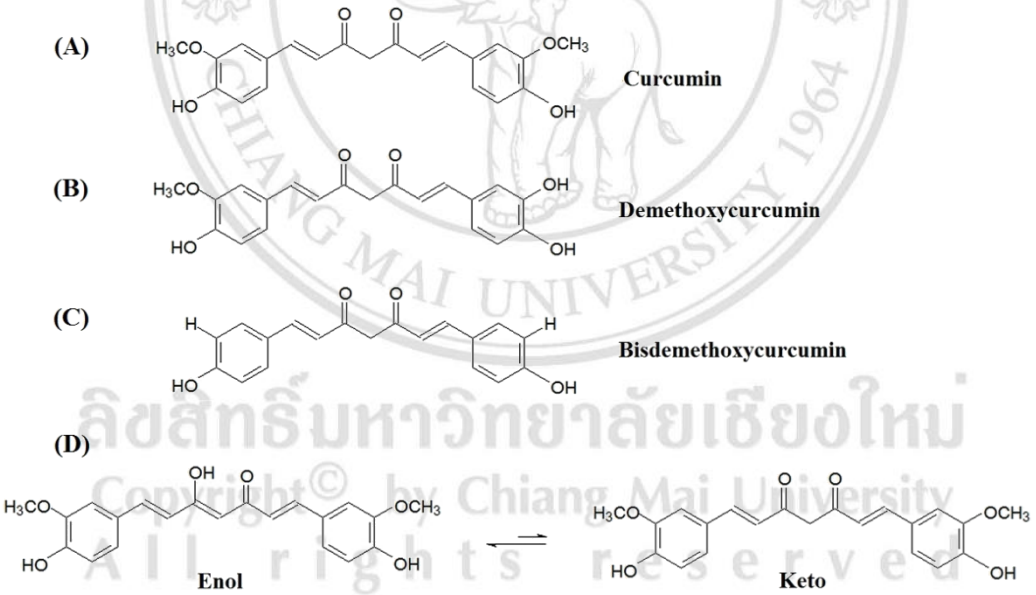

Figure 1.1 Chemical structures of curcumin(A), demethoxycurcumin (B), and bisdemethoxycurcumin (C) and keto–enol tautomeric forms of curcumin (D)

The bis–α,β–unsaturated β–diketone form of curcumin and also of demethoxy-curcumin or bisdemethoxycurcumin exists in a nonpolar environment, whereas the enol tautomer predominates in both aqueous solution and in polar protic or aprotic solvents

as well as in a biological media [39]. Curcumin has a molecular weight of 368.37 and a melting point of 183°C. The appearance of curcumin is a yellow powder. Curcumin is practically insoluble in water. The maximum absorbance of curcumin is 420 nm by UV–visible spectrophotometer.

For many centuries, curcumin in its crude form has been used as spice and dietary supplement as well as component of many traditional Asian medicines [15]. Curcumin has been intensively investigated for its pharmacological activities. The number of publications and clinical trials of curcumin has been increased for decades (Figure 1.2) [40].

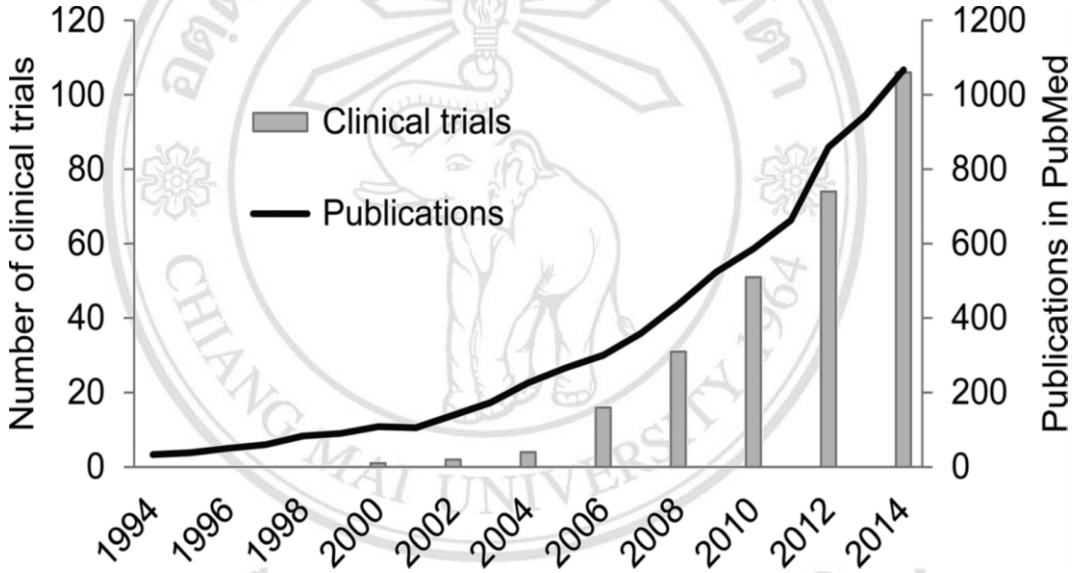

Figure 1.2 The increase publications in PubMed and clinical trials of curcumin [40]

An overview of the different indications for which curcumin has been investigated is shown in Figure 1.3. It has been shown that curcumin exhibits a wide range of pharmacological activities against many chronic diseases including type II diabetes, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease, and atherosclerosis. It also inhibits platelet aggregation, suppresses thrombosis and inhibits human immune-deficiency virus (HIV) replication. Further, curcumin enhances wound healing and protects against liver injury, cataract formation, pulmonary toxicity, and fibrosis [13,19,41-46]. Finally, the anticancer activity of curcumin has been extensively

investigated, and it has been suggested as a potential agent for both prevention and treatment of a great variety of different cancers including gastrointestinal, melanoma, genitourinary, breast, lung, hematological, head and neck, neurological, and sarcoma [12,18,47].

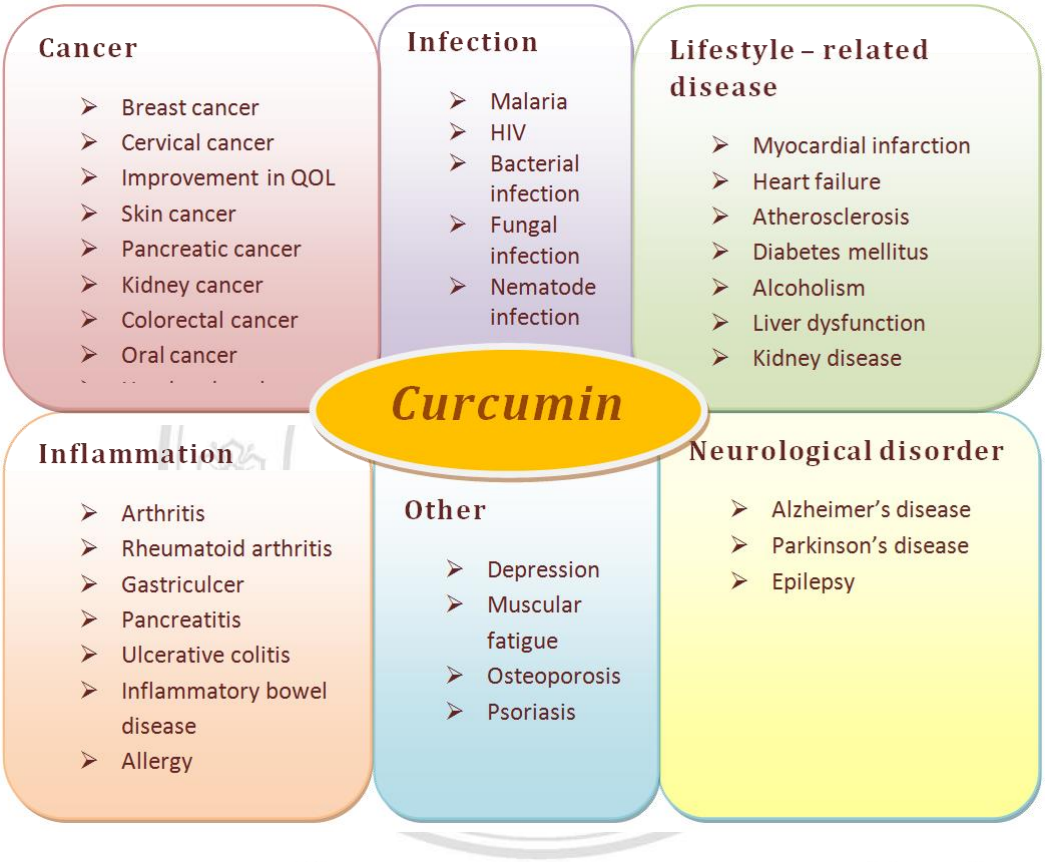

Figure 1.3 The biological activity of curcumin [48]

At a molecular level, curcumin not only inhibits cell proliferation and metastasis, but also induces apoptosis by modulating several pro–inflammatory factors (e.g. interleukin (IL)–1, IL–1β, IL–12, tumor necrosis factor (TNF)–α and interferon (INF)–γ), growth factors (e.g. epidermal growth factor (EGF), hepatic growth factor (HGF) and platelet–derived growth factor (PDGF)), receptors (e.g. epidermal growth factor receptor (EGFR), human epidermal growth factor receptor (HER)–2, IL–8R and Fas–R), transcription factors (e.g. signal transducer and activator of transcription (STAT) 3, nuclear factor (NF)–κB, Wilms' tumor (WT–1), and peroxisome proliferator–activated receptor (PPAR) γ) and protein kinases (e.g., extracellular signal–regulated kinases

(ERK), mitogen–activated protein kinases (MAPK), protein kinase A (PKA) B (PKB), and C (PKC) [49-51]. The overview of molecular targets of curcumin is shown in Figure 1.4 [47].

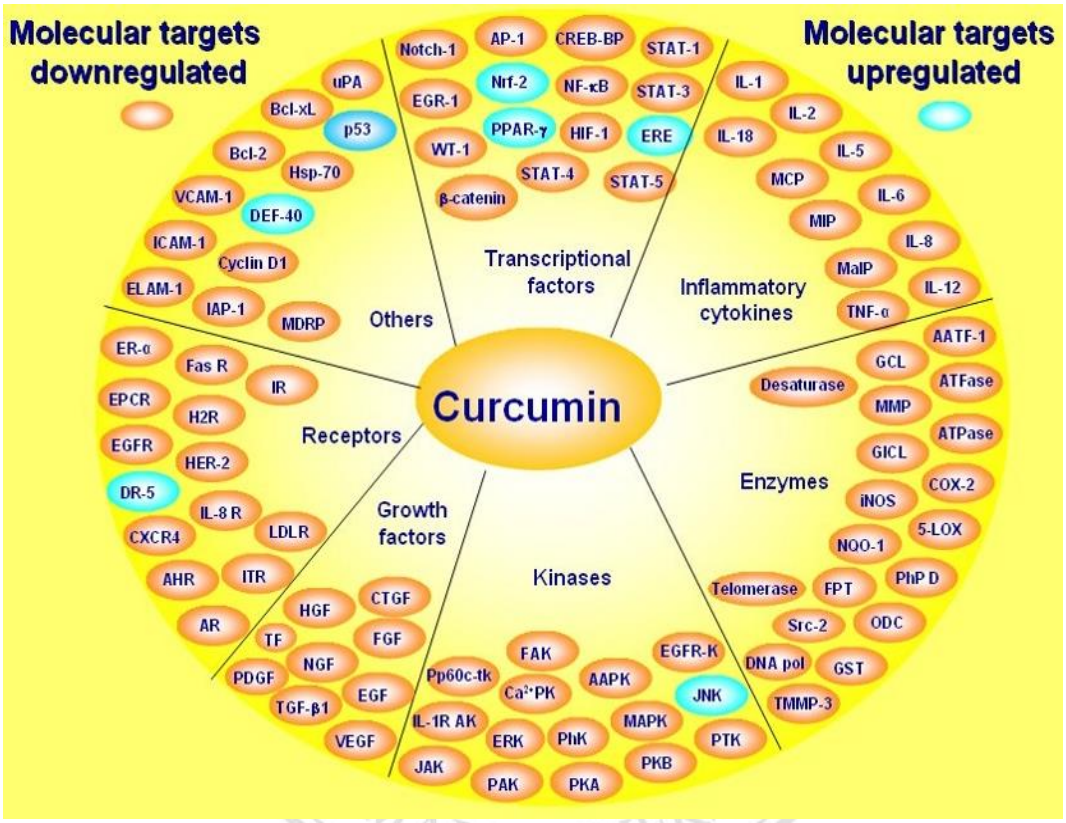

Figure 1.4 The molecular targets of curcumin [47]

The WT1 protein is a 48 – 52 kDa nuclear transcriptional activator or tumor repressor protein [52-54] and is necessary for induction of cell proliferation and differentiation [55-58]. The *WT1* gene was originally found in nephroblastoma (Wilm's tumor), the most common renal solid tumor in childhood. However, the overexpression of *WT1* gene has been reported in leukemia which was significantly higher 1000 and 100000 times than those of in normal bone marrow and peripheral blood cells [59]. Therefore, *WT1* gene and WT1 protein is used as a tumor marker for leukemia. Curcumin was previously reported to induce cell cycle arrest [60-61] and suppress *WT1* gene expression in both primary leukemic cells and continuous cell lines [62-64]. Thus,

in this study, WT1 protein was then used as a target protein model to confirm curcumin activity after loading into HPMA–based polymeric micelles.

In a recent clinical study, it appeared that oral administration of curcumin was well tolerated at doses of 12 g/day which indicates that curcumin is safe [65]. Curcumin can freely pass through cellular membranes due to its lipophilicity (log P = 2.5)[66]. It should however be mentioned that curcumin has a very low aqueous solubility of only 0.6 μg/mL [23]. These characteristics are the cause for its very low bioavailability resulting in suboptimal blood concentrations to achieve therapeutic effects [24,67] as well as the poor absorption, rapid metabolism especially by glucuronidation conjugation, and rapid elimination [67] (Figure 1.5). For instance, in a study in rats reported by Yang *et al.* a maximum serum concentration of 0.36 ± 0.05 μg/mL after an intravenous injection of 10 mg/kg was reached, whereas 500 mg/kg orally administered curcumin gave a maximum plasma concentration of 0.06 ± 0.01 μg/mL, indicating that oral bioavailability was only 1% [68]. Similarly, Shoba *et al.* showed a maximum serum concentration of 1.35 ± 0.23 μg/mL at 1 h after administration of an oral dose of 2 g/kg to rats, whereas healthy man volunteers (weighing 50 – 75 kg) receiving a single dose of 2 g curcumin (4 capsules of 500 mg each) showed an extremely low serum concentration of 0.006 ± 0.005 μg/mL at 1 h [69].

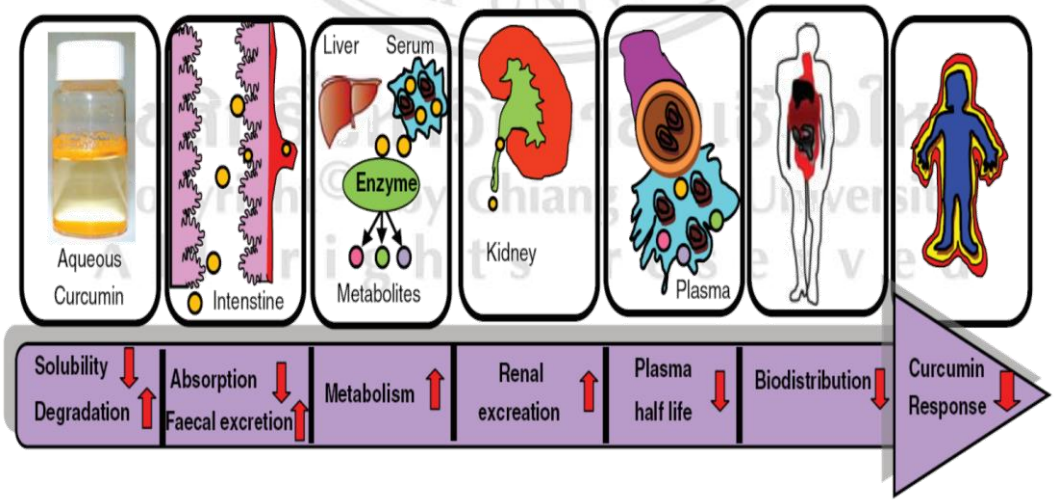

Figure 1.5 The diagram showing the drawback of curcumin solubility and stability [70]

The chemical instability of curcumin under alkaline pH is well documented. Schneider *et al.* published a number of interesting papers in which the degradation of curcumin in aqueous buffer was studied [40]. They also studied in degradation mechanism and identified the formed products. In a recent paper, they proposed a reaction scheme of the oxidative conversion of curcumin *via* 15 intermediate compounds to a deoxygenated bicyclopentadione [71]. They concluded that curcumin does not degrade *via* a hydrolytic process resulting in chain scission as assumed in the older literature [24,25,72], but *via* oxidation yielding a bicyclopentadione final product. They convincingly showed that degradation of 1 mol of curcumin is associated with the consumption of one mol of O_2 [73]. Further, by degrading curcumin in media with $^{18}O_2$ and $H_2{}^{18}O$, they showed that the final degradation product contains one oxygen atom of O_2 and one from water (Figure 1.6) [74].

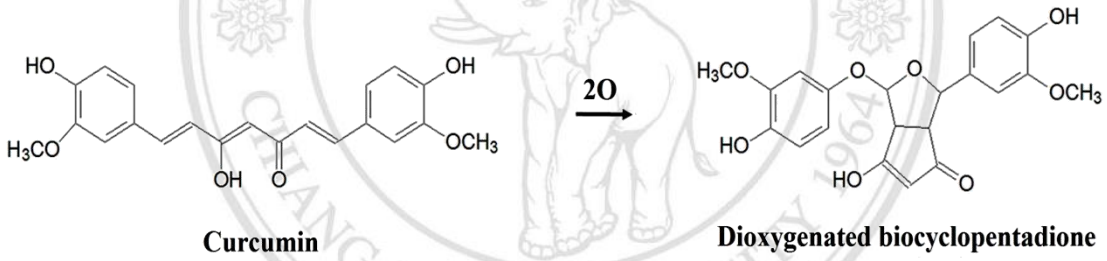

Figure 1.6 Curcumin transforms to a dioxygenated bicyclopentadione *via* autoxidative degradation [74]

The low bioavailability together with the degradation under physiological or alkaline condition is major limitations for clinical application of curcumin. Therefore, particularly nanotechnological approaches have been developed to overcome these problems.

1.3.2 Nanotechnology

In recent years, nanotechnology is well known to apply for medical and pharmaceutical fields particularly in drug delivery systems such as liposomes, polymeric nanoparticles, polymeric micelles, microemulsions, and nanoemulsions. Nanoparticles as nano–scaled drug carriers with size ranging from 10 – 100 nm have

been extensively investigated to optimize the delivery of drugs, recombinant proteins, vaccines, DNA, and RNA [75,76]. These systems alter the pharmacokinetics, bio-distribution, stability, solubility, and drug release of associated drugs [77]. Additionally, they show a low immunogenicity and reduce unwanted side effects [78]. Nanoparticles can also promote tissue–or cell site–specific delivery of drugs by enhanced permeation and retention (EPR) effect as a passive targeting and by antibody conjugation as an active targeting [79,80].

1.3.3 Polymers for pharmaceutical applications

Polymers are macromolecules build up by the linking together of large numbers of small molecules or a so called monomer. The reactions by which they combine together are termed as polymerizations [81]. There may be hundreds, thousands, tens of thousands, or more monomer molecules linked together in a polymer molecule which the molecular weights may reach into the hundreds of thousands or millions.

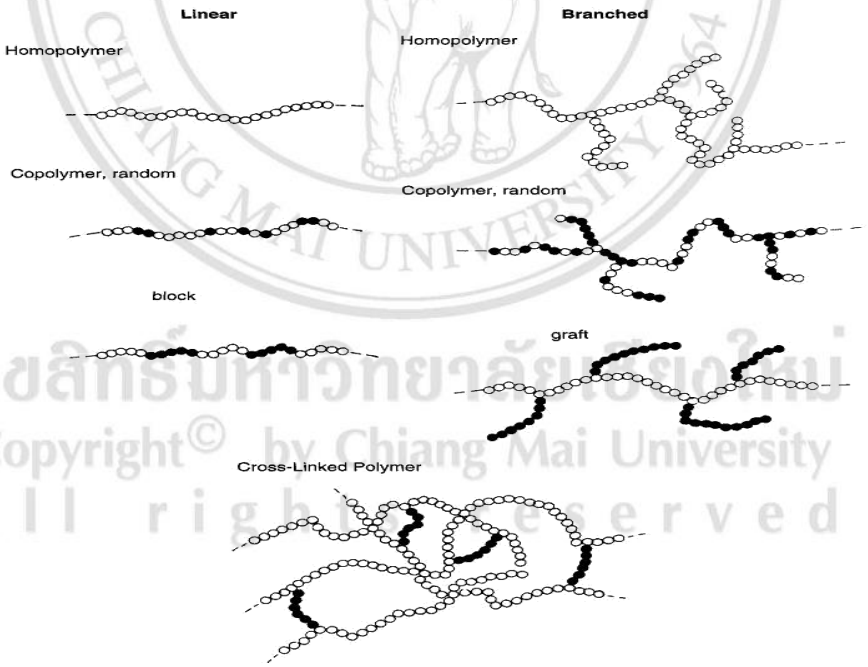

Figure 1.7 Polymer structures
(adapted from http://am medicine.com/2013/12/whatisapolymer.html)

The structure of polymers relevant to pharmaceutical applications is presented in Figure 1.7 as linear polymers, branch polymers, and crosslinked polymers [82]. Linear polymers refer as the one continuous length to form the polymer molecule. Branch polymers are characterized by more than two chain ends per molecule which are side branches of linked monomer molecules protruding from the main polymer chain. Crosslinked polymers are produced by the attachment to each other and formed two or three dimensional network. Polymers that frequently used in pharmaceutical applications as drug loading or as nanocarriers include vinyl polymers, polysaccharides, poly(amino acids), proteins, and poly(ethylene glycol) (PEG). A large number of studies focus on water soluble polymer and amphiphillic polymers which can functionalized their physicochemical properties such as biodegradable polymers (e.g. polylactide copolymers, poly(Ɛ–caprolactone) copolymers, and polyethylene oxide), polyglycolic acid copolymers), thermosensitive polymers (e.g. *N*–isopropylacrylamide (NIPAAm) copolymers, Poly(*N*–vinyl caprolactam) copolymers, and HPMA–lactate co–polymers), and pH responsive polymers (e.g. methacrylic acid (MAAc) copolymers, poly(β–amino ester) (PAE) copolymers, and maleic anhydride (MA) copolymers) [83-89].

Poly[*N*–(2–hydroxypropyl) methacrylamide] (pHPMA) is a hydrophilic polymer currently under investigation for pharmaceutical and biomedical applications because of its good biocompatibility, non–immunogenicity and possibilities for chemical functionalization [90-92]. HPMA has also been investigated as a building block of polymeric micelles. It can serve as hydrophilic part of a micellar stealth corona, while it can also be chemically modified with hydrophobic moieties to serve as a micellar core in which hydrophobic drugs can be solubilized [93].

1.3.4 Polymeric micelles

Polymeric micelles are nano–assembled particles composed of amphiphilic block copolymer which are spontaneously formed above a certain concentration (critical micelle concentration, CMC) (Figure 1.8) [29,31].

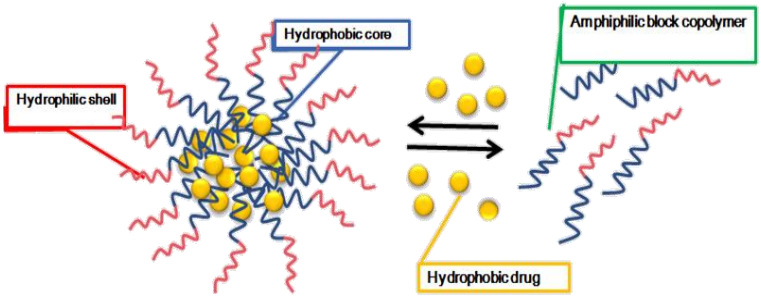

Figure 1.8 Formation and drug loading of polymeric micelles

The formation of polymeric micelles was first observed by Merret *et al*. [94]. Later, the colloidal behavior of micelles formed from block copolymers was studied by Molau [95]. Polymeric micelles have been investigated for many applications especially in improvement of solubility and stability and as drug delivery systems. They were first used as drug carriers by Bader *et al.*[96]. Hydrophobic drugs can be solubilized in hydrophobic core of polymers to enhance their aqueous solubility and their stability [97]. They have a generally small size (10 – 100 nm) and provide a good stealth property to escape from the mononuclear phagocyte system [98,99].

Various polymeric micellar systems have been demonstrated to improve the aqueous solubility and pharmacokinetic of hydrophobic compounds. Soga *et al.* developed a novel polymeric micelles, poly(*N*–(2–hydroxypropyl) methacrylamide lactate) and poly(ethylene glycol) (mPEG–b–pHPMAmDL) for loading paclitaxel [100]. Interestingly, these polymeric micelles can increase paclitaxel solubility up to 2 mg/mL and less toxic than Cremophor EL vehicle. Aliabadi *et al.* studied about methoxy poly-(ethylene oxide)–b–poly(Ɛ–caprolactone) (PEO–b–PCL) which can improve solubility of cyclosporine and pharmacokinetic properties [101]. Bromberg *et al.* showed that copolymers of poly ethylene oxide (PEO) and poly propylene oxide (PPO) Pluronic® polyethers and poly acrylic acid (PAA)were safe and had the ability for enhancing solubility, stability, and bioavailability of anticancer drugs [33]. Moreover, the physical interaction between polymer and drugs including π–π stacking of aromatic groups can provide a good thermodynamic and kinetic stability of polymeric micelles. Carstens *et al.* investigated oligomeric micelles based on mPEG750–b–oligo(Ɛ–caprolactone) with

an aromatic end group [102]. It was shown that the oligomeric micelles enhanced the solubility and stability of the formulation of taxanes. Khonkarn *et al.* developed quercetin loaded PEG–OCL (poly(ethylene glycol)–b–oligo(Ɛ–caprolactone) with naphthoyl or benzoyl end groups which substantially increased solubility of quercetin approximately higher 110 times [103]. Additionally, *N*–(2–benzoyloxypropylmethacrylamide (HPMAm–Bz) or naphthoyl analogue (HPMAm–Nt), with *N*–(2–hydroxypropyl) methacrylamide monolactate with polyethylene glycol as the hydrophilic part of amphiphilic polymers showed the high loading capacity, strong drug retention in the paclitaxel loaded polymeric micelles, and low cytotoxicity of the polymers [104]. Several polymeric micelles with π–π stacking interaction have been investigated to increase the solubility, loading capacity, drug retention, and stability of hydrophobic drugs but not yet in the novel PEG–HPMA–Bz copolymer for curcumin.

1.4. Theory and/or Principle

The incorporation of hydrophobic drugs into the micellar core can be done by chemical conjugation or physical entrapment. The chemical conjugation provides a high drug loading and stability[105-107], but sometimes the chemical conjugation is not always practicable or sometimes impossible when no functional group on the target molecule for conjugation is available. Further, covalent bonds in the core of polymeric micelles are hardly hydrolyzed or cleaved by enzymes because of the steric hindrance leading to ineffective therapeutics [108,109]. Therefore, physical entrapment of drugs is preferred to prepare drug–loaded micelles [110]. But in many case the drug is insufficiently retained in the micellar formulation. The introduction of π–π stacking between aromatic groups of the drug and the polymer has been exploited to enhance solubility (or drug loading) and drug retention. In this present study, It was hypothesized that a synthesized block copolymer PEG and HPMA modified with aromatic benzoyl groups (PEG–HPMA–Bz) might enhanced the aqueous solubility and stability by π–π stacking interaction (Figure 1.9) and provide the novel curcumin–loaded polymeric micelles which is underlined as a safe and effective drug delivery systems for further pharmaceutical and clinical development for cancer treatment.

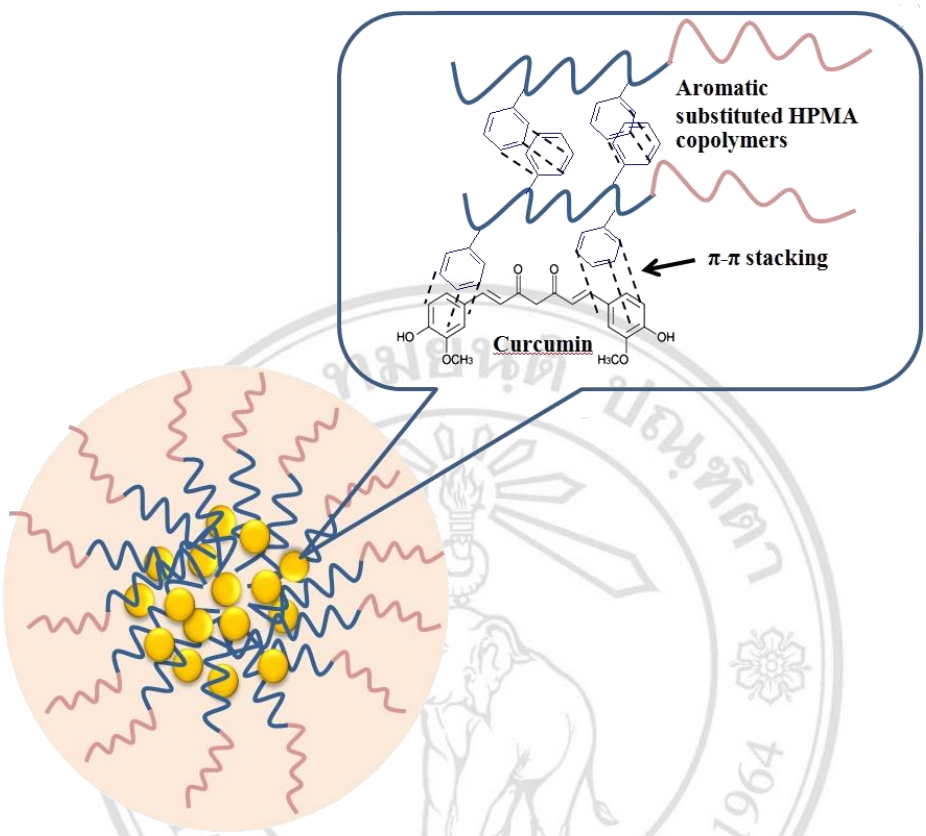

Figure 1.9 π–π stacking interaction between aromatic groups of curcumin and polymer

1.5. Scope of the Study

	1st year	2nd year	3rd year	4th year	5th year
Determination of biological activity of curcumin	↔				
Determination of physicochemical characteristics of curcumin	↔				
Synthesis and characterization of polymers		↔			
Preparation of polymeric micelles		↔			
Characterization ofcurcumin–loaded polymeric micelles		↔	↔		
In vitro release study of curcumin–loaded polymeric micelles			↔		
Interaction of curcumin–loaded polymeric micelles with blood components				↔	
Cytotoxicity of curcumin–loaded polymeric micelles by MTT assay				↔	
Effect of curcumin–loaded polymeric micelles on cell cycle arrest by flow cytomeetry				↔	
Effect of curcumin–loaded polymeric micelles on Wilms' tumor 1 (WT1) target protein by Western blot analysis				↔	

1.5 Scope of the Study (continued)

	1st year	2nd year	3rd year	4th year	5th year
Stability of curcumin–loaded polymeric micelles				←	→
Thesis and manuscript writing/ Examination					↔

1.6. Expecting Benefit and/or Goal

1.6.1 Acquirement of technology to synthesize copolymers

1.6.2 Acquirement of technology to enhance solubility of curcumin by using polymeric micelles

1.6.3 Acquirement of technology to enhance stability of curcumin using polymeric micelles

1.6.4 Acquirement of factors affecting curcumin–loaded polymeric micelles

1.6.5 Benefits of curcumin–loaded polymeric micelles in pharmaceutical applications