Selective Inhibition of Osteosarcoma Cell Functions Induced By
Curcumin-Loaded Self-assembled Arginine-Rich-RGD Nanospheres

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ABSTRACT

Osteosarcoma is the most frequent primary bone cancer today. Chemotherapy mostly used for the treatment against osteosarcoma can induce high toxicity. Therefore, the objective of this in vitro study was to develop a treatment against osteosarcoma with higher selectivity towards osteosarcoma cells and lower cytotoxicity towards normal healthy osteoblast cells.

Curcumin (or diferuloylmethane) has been found to have anti-oxidant and anti-cancer effects by multiple cellular pathways. However, with its polyphenol groups, it has low water solubility and a high degradation rate in alkaline conditions.

Self-assembled amphiphilic peptides have various applications as novel nanoscale biomaterials including hydrophobic drug delivery. In this study, the amphiphilic peptide C18GR7RGDS was used as a curcumin carrier in aqueous solution. This peptide contains a hydrophobic aliphatic tail group leading to their self-assembly by hydrophobic interactions, as well as a hydrophilic head group composed of arginine-rich and an arginine-glycine-aspartic acid (RGD) structure, which may lead to efficient cell internalization by macropinocytosis and targeting for the overexpressed αvβ3 integrins on cancer cells. Through the characterization of transmission electron microscopy (TEM), the self-assembled structures of spherical amphiphilic nanoparticles (APNPs) with diameters of 10-20 nm in water and phosphate buffer saline (PBS) were observed, but this structure opened up when the pH value was reduced to 4. Using a method of co-dissolution with acetic acid and dialysis tubing, the solubility of curcumin was enhanced and formed a homogeneous solution with the help of the presently designed
APNPs. The successful encapsulation of curcumin in APNPs was then confirmed by Fourier transform infrared (FT-IR) and X-ray diffraction (XRD) analysis. Also, the cytotoxicity and cellular uptake of the APNP/curcumin complexes on both osteosarcoma and normal osteoblast cell lines were evaluated by MTT assays and confocal fluorescence microscopy. Most importantly, the curcumin-loaded APNPs exhibited a significant selective cytotoxicity against MG-63 osteosarcoma cells (15% of viability) compared to normal osteoblasts (more than 50% of viability). In this manner, it was demonstrated for the first time that APNPs can encapsulate hydrophobic curcumin in their hydrophobic cores, and the curcumin-loaded APNPs could be an innovative drug for the selective inhibition against osteosarcoma cells than osteoblasts.
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1.0 Introduction

1.1. Motivation and Background

Osteosarcoma is the most frequent primary malignant bone cancer, comprising 30% of all the cases of bone cancer. The chemotherapy mostly used for the treatment against osteosarcoma can induce high toxicity [1,6]. Therefore, the objective of this in vitro study was to develop a treatment against osteosarcoma with higher selectivity towards osteosarcoma cells and lower cytotoxicity towards normal osteoblast cells.

Curcumin (or diferuloylmethane) has been found to have anti-oxidant and anti-cancer effects by multiple cellular pathways. However, with its polyphenol groups, it has low water solubility and a high degradation rate in alkaline conditions [50-52].

Self-assembled amphiphilic peptides have various applications as novel nanoscale biomaterials including hydrophobic drug delivery [73-75]. In this study, the amphiphilic peptide C18GR7RGDS was used as a curcumin carrier in aqueous solution. This peptide contains a hydrophobic aliphatic tail group leading to their self-assembly by hydrophobic interactions, as well as a hydrophilic head group composed of arginine-rich and arginine-glycine-aspartic acid (RGD) structure, which may lead to efficient cell internalization by macropinocytosis [128] and targeting overexpressed ανβ3 integrins on cancer cells [101,119].

1.2 Objectives and Scope

The main objective of this study was to develop a nano-scale drug that has
selective cytotoxicity against the MG-63 osteosarcoma cell line than healthy, non-cancerous human osteoblast cell line in vitro. The specific aims include:

1. To investigate the morphologies of C18GR7RGDS self-assembled amphiphilic peptide nanoparticles (APNPs) in deionized water, phosphate buffer saline (PBS), and acetic acid at pH=2, 4, and 6.

2. To increase the water-solubility and bioavailability of curcumin by utilizing C18GR7RGDS self-assembled amphiphilic peptide nanoparticles (APNPs) and to characterize the morphologies of APNPs after curcumin encapsulation.

3. To observe the increased solubility capability of curcumin after it is entrapped by APNPs in aqueous solution, as well as the solubility of the dry powder of lyophilized curcumin-loaded APNPs.

4. To analyze the possible chemical interactions after the drug loading process by Fourier Transform Infrared (FT-IR) spectroscopy and X-ray Diffraction (XRD) study.

5. To evaluate the cytotoxicity as well as the cellular uptake of curcumin-loaded APNPs by both the MG-63 osteosarcoma cell line and human osteoblast cell line, and compare the selective cytotoxicity induced by curcumin-loaded APNPs with plain curcumin suspended in PBS as well as curcumin dissolved in dimethyl sulfoxide (DMSO), an organic solvent that can be used for dissolving hydrophobic anti-cancer drugs.
2.0 Critical Literature Review

2.1 The Epidemiology of Osteosarcoma

Osteosarcoma is the most frequent primary malignant bone cancer, comprising 30% of all the cases of bone cancer. It often arises from in the metaphyses of long bones of adolescents that have the highest growth potential [1]. Osteosarcoma was characterized by giant cell tumors that could enlarge aggressively and extend through the bone cortex in the surrounding soft tissue [2]. The localization of osteosarcoma is mostly in the metaphyseal growth plates of long bones including the femur (incidence rate 42%), tibia (incidence rate 19%), and humerus (incidence rate 10%), as well as other possible localizations in the skull or jaw (incidence rate 8%), pelvis (incidence rate 8%) and ribs (incidence rate 1.25%) (Figure 2.1). Osteosarcoma occurs by a bimodal age distribution having two peaks. The first peak of incidences of osteosarcoma in adolescents is in 10-14 year-olds when pubertal growth starts, and the second peak is in adults at the age of 65 or older. In the United States, there are approximately 400 cases of osteosarcoma diagnosed every year [6]. Interestingly, patients having osteosarcoma before the age of 15 were taller than the general population [3,4].
The survival rate of osteosarcoma has improved from historic rates up to 20% to above 63% after 1974 due to the advent of multi-agent chemotherapy. Before 1970, the only surgical treatment for osteosarcoma was amputation, causing 80% of patients to die from subsequent metastasis, which mostly occurred in the lungs. After the adoption of multi-agent chemotherapy, there was a significant increase of survival rate reported for the years 1974-1994 in which there was a lower presence of metastasis and local recurrence. However, there was no significant improvement on the survival rate of osteosarcoma over the last several decades. According to a report by The National Cancer Institute, the survival rate of patients under the age of 45 suffering from osteosarcoma was nearly constant at 65% from 1975-2000. Thus, the development for safer, novel, and more targeted therapies is still necessary [1,5,6].

2.2 Factors of Osteosarcoma

2.2.1 Chromosomal Aberrations

An increased risk for osteosarcoma is frequently documented in patients with
abnormal genetic conditions including hereditary retinoblastoma and Li–Fraumeni syndrome, which are associated with the mutation of the retinoblastoma gene Rb1 on chromosome 13q14 and p53 tumor suppressor gene, respectively. Several chromosomal abnormalities of osteosarcoma tissue have been observed by the application of comparative genomic hybridization. The gains of chromosome 1p, 2p, 3q, 5q, 5p, and 6p and losses of 14q and the losses of 14q, 15q, and 16p were found. In the regions of chromosome 21, the 21q11.2~21 region was mostly found absent in osteosarcoma [6]. Additionally, Smida et al. reported that the most common genomic abnormalities in osteosarcoma included the amplification of chromosomes 6p21, 8q24 and 12q14, and the loss of 10q21.1 [7].

2.2.2 Mutation of Tumor Suppressor Genes

Many studies have reported that the occurrence of osteosarcoma could be related to p53 and retinoblastoma (Rb) tumor suppressor gene dysfunction [5,8]. These tumor suppressors serve as inhibitors of abnormal cell cycle progression, and lead to impaired DNA repair and cell cycle arrest. Between each cell cycle stage, there are checkpoints that regulate the cell cycle system. DNA damage, which is mostly caused by exposure of environmental insults including radiation [1] and chemical agents [8-10], can be detected in the checkpoints. The protein products from tumor suppressor genes can therefore inhibit the irregular cell cycle in these checkpoints. However, the inactivation of tumor suppressors can lead to uncontrolled cell proliferation, which is one of the characteristics of cancer. Almost all types of cancer are caused by a disregulation of cell proliferation in
the G1 phase which is the interval between mitosis and the onset of DNA replication.

Rb is one of the tumor suppressors whose primary function is to block cell cycle progression until mitogenic stimulation results in its inactivation. By binding with a family of essential transcription factors known as E2F, Rb can regulate cell cycle progression. If external signals occur, Rb is phosphorylated and the cell can thus pass through the cell cycle. The defective Rb can usually develop inappropriate cell cycle and cancer including osteosarcoma. In addition, p53 is also a transcription factor that functions as an activator of various target genes such as p21. If p53 is activated, the cell cycle arrest and apoptosis are thus activated with higher levels of cell-killing proteins (i.e., Bax, Apaf-1, and CD95). In healthy cells, a partner protein Mdm2 regulates p53, limiting the low level of p53 so that the cell cycle can operate normally. Otherwise, when oncogene activation occurs, Mdm2 could be sequestered in the nucleolus, and then p53 is activated, promoting apoptotic cell death [11]. Chandar et al. reported that the first intron of the p53 gene is mutated in many osteosarcoma cell lines, which results in the loss of p53 expression and abnormal DNA repair mechanisms [12].

2.2.3 Overexpression of Transcription Factors

Transcription factors are proteins that can regulate the transcription process. By binding to specific promoter sequences or blocking the RNA polymerase interaction on double-stranded DNA, the transcription factors can initiate or cease the transcription process in which messenger RNA (mRNA) sequences generate. For many cancer cells lines including osteosarcoma, excessive production of transcription factors occur as a
result of gene rearrangement, leading to irregular cell proliferation [5].

The activator protein 1 (AP-1) complex is one of the transcription factors that stimulate cell differentiation, proliferation, apoptosis and oncogenic transformation. AP-1 is a heterodimeric protein that consists of different types of dimers composed of members of the Fos (c-Fos, FosB, Fra-1, Fra-2) and Jun (c-Jun, JunB, JunD) proteins. For the members of Jun proteins, c-Jun is a positive regulator of cell proliferation and negative regulator of p53 gene since it can stimulate the G1 phase to S phase in the cell cycle by inducing cyclin D1 that is overexpressed in cancer cell lines, and c-Fos and FosB can also enhance the stimulation of cyclin D1 expression and further induces cells to enter the S phase from G1 phase. In contrast, JunB and JunD proteins have negative effects on cell cycle progression due to the fact that JunB inhibits the expression of cyclin D1 and induce the expression of cell cycle repressing protein p16, and JunD can inhibit entry into the S phase (Figure 2.2). In normal osteoblast cells, the levels of all Fos and Jun proteins are high during the cell proliferation phase, but during the mineralization and extracellular matrix production phase, the proteins including JunD become the dominant components of the AP-1 in fully differentiated osteoblasts [13]. However, Franchi et al. reported that c-Jun and c-Fos might be overexpressed in high-grade osteosarcoma cell lines, thus, were characterized by a higher proliferation rate [14]. Additionally, Wang et al. also stated that significant levels of c-Fos with the co-expression of c-Jun was necessary for malignant osteosarcoma development, and a higher level of AP-1 might enhance osteoblast progression and transformation to bone tumors by the observation of in vivo studies [15].
The nuclear factor kappa B (NF-kB) is also an important transcription factor that could initiate and promote cancer. NF-kB is kept inactive in the cytoplasm when inhibited by IkB kinase. When normal B-lymphocytes interact with antigens, or stimulated either by oxidative stress or free radicals, the NF-kB inhibitor IkB is phosphorylated, causing a higher level of active NF-kB, and thus fostering an inflammatory environment by various cytokines that favor malignant transformation [16]. The active NF-kB transcription factor can translocate into the nucleus, and positively regulate different gene products related to anti-apoptosis, pro-inflammatory, and proliferation. The pro-inflammatory gene products include cyclooxygenase-2 and the anti-apoptosis protein cyclin D1 and Bcl-2 [17, 18]. As a result, apoptosis is blocked under high levels of active NF-kB, which could potentially benefit cancer activity. The increased activation of NF-kB is observed in many cancer cell lines, and involved in anti-apoptosis, tumor promotion and metastasis [19]. More importantly, since the survival of most of the tumor cells is dependent on NF-kB, the active NF-kB was observed in many cancer cell types. Aggarwal described that the
inflammation and tumorigenesis processes were analogous to “fuel on fire” as the pro-inflammatory and anti-apoptosis effects could exaggerate cancer promotion [21]. The active NF-kB level can serve as a predictor for tumorigenesis as well as a potential agent for targeting cancer cells by therapeutic agents that can downregulate active NF-kB and can, thus, down-modulate tumorigenesis [20, 21].

2.3 Chemotherapy Treatments For Osteosarcoma

Current treatments for osteosarcoma can differ in the level of tumorigenesis. For low-grade sarcomas, surgical excision is the main treatment strategy, but for high-grade bone sarcomas, chemotherapy is mostly required before and after the surgical extirpation of the tumor in order to improve the systemic control and decrease the degree of reoccurrence of the osteosarcoma. The neoadjuvant (presurgical) treatments are achieved by 3 to 4 cycles, and the duration of adjuvant (postoperative) chemotherapy is dependent on the degree of tumor necrosis found at the time of surgery [1].

A number of therapeutic agents are currently used in the treatment of osteosarcoma. They are comprised of doxorubicin, high-dose methotrexate with leucovorin rescue, cisplatin, and oxazaphosphorines (cyclophosphamide and ifosfamide). In the multi-agent chemotherapies that are administered, various combinations of these agents may be applied to minimize tumor size and achieve a safer surgical resection [6].
2.3.1. Doxorubicin

Doxorubicin (or Adriamycin) is the most widely used agent for chemotherapy due to its significant anti-tumor efficacy since it can intercalate into DNA and induce topoisomerase II-mediated single- and double-strand breaks in the DNA [6]. It is an anthracycline, tetracyclic aglycone, water-insoluble compound with red color extracted from cultures of *Streptomyces peticetius var. caesius*. Middleman et al. reported that all of the 67 patients in their study were administrated doxorubicin intravenously and then were observed to have rapid tumor regression during early treatment with doxorubicin. Nevertheless, a series of toxic responses induced by doxorubicin was also observed. Specifically, 100% of responsive patients had alopecia (hair loss), 21% had nausea and vomiting, and 6% had phlebitis [22]. Moreover, many studies reported that high dosages (at least 550 mg per square meter of body-surface area) of doxorubicin treatment can lead to acute cardiac failure due to dose intensity and cumulative doses. Cardiac abnormalities induced by doxorubicin can occur within either one year or more than ten years after chemotherapy [23-26]. Longhi et al. reported that even over two decades after the doxorubicin chemotherapy, 13 in 755 patients who were treated from 1983 to 2000 developed a clinically symptomatic cardiac toxicity, six of them were dead due to the cardiac failure and seven of them required a heart transplant [27]. The cause of doxorubicin-induced cardiomyopathy might be the generation of hydroxyl free radicals during anthracycline metabolism by NADH dehydrogenase, and these radicals may increase oxidative stress and damage the mitochondria of myocardial cells [24, 28].
2.3.2 High-Dose Methotrexate With Leucovorin Rescue

Methotrexate has been used as a chemotherapy drug since the 1970s. Methotrexate turns off DNA and RNA synthesis by binding stoichiometrically and irreversibly to dihydrofolate reductase which is necessary for cellular folic acid uptake, thus, inducing cell death. On the other hand, leucovorin (5-formyl tetrahydrofolate) serves as an antidote to methotrexate by converting itself to 5,10 methylene tetrahydrofolate and 5-methyl tetrahydrofolate in cells [29]. Methotrexate concentration is measured using by assessing serum levels. Methotrexate is administrated intravenously over 4 to 6 hours, and its mean maximum serum level is over 700 to 1000 µM. During the first 24h after methotrexate injection, leucovorin is injected for the successful completion of therapy. The optimal dosage intensity of methotrexate is dependent on whether or not it is used as a single-agent therapy. When administrated as an intensified course as a single-agent, the optimal dosage intensity is 4-8 doses at a 10- to 14-day interval; when combined with other chemotherapy drugs (i.e., doxorubicin, cisplatin and ifosfamide), the interval between courses may increase to 3-4 weeks [6, 29, 30]. In terms of the efficacy of methotrexate, a number of studies have reported that the administration of methotrexate as the only chemotherapy agent after tumor extirpation could improve the survival rate to 40%, whereas the survival rate could rise to over 65% when methotrexate is administrated with other chemotherapy agents [31-34]. Most recently, Sun et al. reported that higher dosage intensities of methotrexate could induce a greater 5-year event-free survival rate in 2,275 patients from 1976 to 2006 [35]. However, it was reported that increasing the administered concentration of methotrexate at the tumor site was not
significant compared to intravenous administration [36].

Prerequisites for a patient to take a high-dose of methotrexate therapy include normal renal and hepatic function, a normal hemogram, and absence of infection. Regarding the toxicity of methotrexate, the precipitated drug in the renal tubules and incomplete metabolism would cause kidney and liver failure and gastrointestinal mucosal ulceration [6].

2.3.3 Cisplatin

Cisplatin is a platinum chemical and one of the most useful chemotherapy agents. Its chemical structure consists of a platinum atom in the center that is covalently conjugated with two chlorine atoms and two ammonia groups in a *cis* configuration [37]. When the intracellular concentration of cisplatin is about one-thirteenth compared to the extracellular concentration, cisplatin molecules become active, leading to an aquation reaction and replacing the one or two chlorine atom with water or a hydroxyl group. The resulting compound tends to function as a DNA adduct when it is added to the same DNA strand, or as a DNA cross-link when it is linked to two different DNA strands. Cisplatin also has a tendency to link to N-7 positions of adenine and guanine even if it can bind to all DNA bases. As a result, DNA synthesis in affected cancerous cells is inhibited (Figure 2.3) [38].
Jaffe et al. demonstrated that an intra-arterially administrated dosage of cisplatin was initially 150 mg/m$^2$ over 2 hours at 3-week intervals in four courses of preoperative treatment for osteosarcoma [39]. Later on, the dosage was reduced to 120 mg/m$^2$ over 4 hours, combining with 95 mg/m$^2$ of doxorubicin concurrently over 24 hours. When cisplatin was administrated intra-arterially at 150 mg/m$^2$, the highest concentration in the local vein was from 9-10 µg/ml at 60-90 minutes, but in the peripheral vein, the highest concentration was 1.7-3.9 µg/ml at 30-120 minutes. The efficacy of cisplatin was related to its local concentration. Less than 60% of tumor destruction was induced for concentrations lower than 12 µg of cisplatin per gram of weight of the patient’s body (µg/g), and 60%-100% of tumor destruction was achieved for concentrations of 17-40 µg/g [6].

Nonetheless, the toxicity caused by cisplatin is significant. Ruiz et al. reported that in 54 patients (5-18-years-old) with primary bone tumors, bilateral hearing loss occurred in all of them in the 6-year observation after treatment with 604 cumulative courses of cisplatin [40]. In terms of the ototoxicity induced by a high-dose of cisplatin on humans,
Pollera et al. reported that 75% of patients receiving at least two courses of cisplatin treatment had moderate to severe hearing loss, and the ototoxicity could not be reduced by the use of hypertonic saline or vigorous hydration [41]. Furthermore, nephrotoxicity is one of the most frequent toxic reactions caused by cisplatin. Ries et al. showed that cisplatin caused significant nephrotoxicity in a dose-dependent manner, and the renal insufficiency could be prevented by hydration and forced diuresis [42]. In addition, hypomagnesaemia, hypocalcaemia, gastrointestinal toxicity and myelosuppression can also be triggered by cisplatin treatment [43].

2.3.4 Oxazaphosphorines

The oxazaphosphorines including cyclophosphamide (CY) and ifosfamide (IFO) are alkylating agents that are activated by hepatic microsomes [6]. For CY, it is transformed to active metabolites consisting of aldophosphamide and phosphoramid mustard in the liver. These metabolites can bind to DNA strands, thus, inhibiting cell division and causing cell death. When CY is administrated as a chemotherapy agent, etoposide is combined to enhance the efficacy by synergistic interaction [44]. Similarly, IFO must be activated by the oxidase system in the liver before demonstrating alkylating activity, although it shows a slower metabolism process for activation since it differs from cyclophosphamide in the placement of its chlorethyl groups [45].

Both CY and IFO can induce high risks of hemorrhagic cystitis. The urotoxicity of these oxazaphosphorines is associated with the metabolite acrolein. According to Brock et al., acrolein and renal elimination of 4-hydroxy metabolites could induce a
dose-dependent urotoxicity after the administration of oxazaphosphorines. However, this bladder toxicity is independent of the alkylating activity [45,46]. Furthermore, it has been shown that the use of alkylating agents including CY for cancer during childhood or adolescence significantly induces higher risks for early menopause during the ages of cancer survivor at 21 to 25, and in the age of 31, 42% of female cancer survivors had menopause compared to those patients that did not receive alkylating agents [47]. Palmert et al. also reported that using IFO as a chemotherapy agent could increase the risk of premature ovarian failure [48].

2.4 Curcumin

Since the commonly used chemotherapy agents that are discussed above are shown to be toxic to both tumor and normal tissues, it is necessary to develop new drug that has selective toxicity against the osteosarcoma tumor tissue. Curcumin (chemical name: (E,E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6- heptatriene -3, 5 dione, Figure 2.4) is a component of turmeric. It is generally extracted from the roots of *Curcuma Longa*, which grows naturally throughout the Indian subcontinent and in tropical countries, especially in Southeast Asia. The yellow color of the rhizome of *Curcuma Longa* can be attributed to non-volatile compounds, especially curcumin.

Curcumin is one of the components of curcuminoids. The curcuminoids constitute approximately 5% of the powdered extract of *Curcuma Longa*, which consists of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin. In the late 19th century, high purity curcumin has been prepared, and it has been used as a coloring agent,
preservative, flavoring, and food additive in modern times. More recently, many scientific researchers have been focusing on its clinical use and potential on cancer inhibition by molecular and cellular pathways [49].

![Figure 2.4 Chemical structure of curcumin](image)

The Food and Drug Administration (FDA) in the U.S.A. has recognized the safety of curcumin and turmeric products. The Expert Joint Committee of the Food and Agriculture Organization/World Health Organization (FAO/WHO) has characterized the daily uptake of curcumin in the range among 0.1-3 mg/kg as safe. Importantly, according to the systematic preclinical studies funded by the Prevention Division of the U.S. National Cancer Institute, adverse effects of curcumin administrated lower than 3.5g/kg-body within 90 days were not detected in rats, dogs, or monkeys [50].

### 2.4.1 Chemical Properties of Curcumin

With the polyphenol chemical structure, curcumin is in orange-yellow color crystalline form and insoluble in water [51]. The curcumin molecule has two methoxylated phenol groups, and can appear in an enolate form as well as bis-keto form by an equilibrium condition. The keto form predominates in acidic conditions, neutral conditions and when in a solid phase, whereas the enolate form under alkaline conditions dominates where the phenol groups of the molecule serve as an electron donor (Figure
In basic conditions, curcumin is unstable and mostly degraded to trans-6-(4’-hydroxy-3’-methoxy –phenyl)-2,4-dioxo-5-hexanal, vanillin, feruloylmethane, and ferulic acid. As a result, curcumin is less yellow and redder in alkaline condition [49]. The pKa values for the dissociation of the three protons in the curcumin molecule have been determined to be 7.8, 8.5, and 9.0 [52]. Wang et al. investigated curcumin degradation in phosphate buffers at pH 7.2 in different physiological conditions in vitro. They showed that 90% of curcumin degraded within 40 minutes and 60 minutes in 0.1M phosphate buffer and serum-free cell culture medium, respectively. However, in medium containing 10% of serum, less than 20% of curcumin was degraded since the albumin in the serum prevented degradation. Also, this degradation reaction can be blocked by the presence of antioxidants (i.e., vitamin C, N-acetylcysteine, and glutathione). On the other hand, curcumin is much more stable in the acidic conditions [53]. Furthermore, curcumin can decompose under light exposure [54, 55], and Wang et al. also reported that almost 5% of curcumin degraded after exposure to sunlight [53].

Figure 2.5 Chemical structures of curcumin at different pH (adopted from [49])
Moveover, the free radical scavenging activity of curcumin is also of interest. In a study conducted by Masuda et al., the anti-oxidant effects and mechanisms of curcumin was investigated. They showed that the polyphenol groups of curcumin could couple with lipid hydroperoxyl and peroxyl radicals produced by biomolecules, producing inactive radicals after an intramolecular Diels-Alder reaction [56].

2.4.2 Anti-Cancer Effects of Curcumin

Curcumin has been shown to have potential as a chemotherapeutic agent against many types of cancers since it possesses pleiotropic anti-carcinogenesis efficacies. By affecting various molecular and cellular pathways, curcumin can play important role in anti-oxidant, anti-inflammatory, anti-bacterial, wound healing, and especially anti-cancer activities. In terms of its anti-cancer activities, curcumin is able to target cellular processes including gene expression, transcription, proliferation, and progression [49]. Additionally, Wilken et al. demonstrated that curcumin could not only show its anti-proliferative effects towards many types of cancer by inhibiting NF-kB and its downstream gene products, but also affect various growth receptors and cell adhesion molecules in tumor growth [57].

2.4.2.1 Effects On NF-kB and AP-1 Transcription Factors

As mentioned earlier, NF-kB is a transcription factor that regulates the transcription of genes involved in inflammation, cell proliferation and anti-apoptosis effects. NF-kB expression is stimulated by a serious of stressful stimulus including UV-irradiation, free radicals, cytokines, and infectious antigens [49, 57].
The inhibitory effects of curcumin on NF-kB were proven by a number of studies. For example, Brennan et al. found that antioxidant agents especially curcumin could inactivate NF-kB by direct modification and inhibit the degradation of the NF-kB inhibitor, IκBa, thus retaining NF-kB in the cytosol [58]. Furthermore, Jobin et al. concluded that curcumin could block the upstream kinase signal inducing NF-kB and IKK, and then potently inhibit NF-kB activation [59]. Subsequently, gene products of NF-kB including cyclooxygenase-2 (COX-2), cyclin D1, interleukin-6, Bcl-X and Bcl-2 are down-regulated by curcumin since the NF-kB bound by its inhibitor cannot transport into the nucleus to activate DNA transcription.

Bharti et al. reported that for human multiple myeloma cells, curcumin can decrease the expression of various gene products regulated by NF-kB, and suppress cell proliferation by arresting the cell cycle at the G1/S phase. In a study investigating the programmed cell death of osteosarcoma cells induced by curcumin treatment, Jin et al. demonstrated that curcumin could induce apoptosis of U2OS osteosarcoma cell lines in vitro. In the same study, the level of pro-apoptotic proteins including Bad, Bax, and Bak increased with the increasing concentrations (0-100 µM) of curcumin, and the increase of curcumin concentration could suppress the anti-apoptosis protein Bcl-2 [61].

On the other hand, AP-1 is also a transcription factor that regulates the cellular pathway to tumorigenesis. Similar to NF-kB, the activation of AP-1 can be stimulated by oxidative stress, UV-irradiation, and pro-inflammatory cytokines [57]. The c-Jun protein, which is one of the subgroups of AP-1 dimers, plays an important role in cell life and death by regulating the level of cyclin D1 and the expression of p53, p19, p21, and p16.
tumor suppressor genes. The c-Jun protein can also promote cell proliferation via repression of the p53 tumor suppressor gene [62]. Although Strimpakos et al. reported that the role of curcumin in AP-1 regulation was uncertain [49], Park et al. suggested that curcuminoids including curcumin could suppress the formation of the DNA of the Jun-Fos complex, leading to a decreased level of AP-1 protein (Figure 2.6). They also found that curcumin can interfere with c-Jun/DNA binding, reducing transcription of its gentic products, suggesting that curcumin could have anti-cancer potentials [63].

![Figure 2.6 Mechanism of curcuminoids inhibition on AP-1 (adopted from [63])]
increase and cytochrome c can leak into the cytoplasm. Cell death is also triggered after cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1). Therefore, the level of the p53 transcription factor can positively regulate Bax levels, initiating intrinsic apoptosis (Figure 2.7) [58].

![Figure 2.7 Intrinsic apoptotic pathway induced by p53 activation (adopted from [58])](image)

Curcumin has been shown to up-regulate p53 expression in various cancer cell lines. For example, Choudhuri et al. found that curcumin could increase the expression of p53 dramatically in mammary epithelial carcinoma cells (MCF-7) in the G2 phase of the cell cycle, while untreated MCF-7 cells had low p53 expression. On the other hand, curcumin only increased the level of p53 expression in normal mammary epithelial (NME) cells by a low extent. In the same study, an increased level of cytochrome c was observed in MCF-7 cells after curcumin treatment, but the release of cytochrome c from mitochondria was not observed in NME cells [64]. In addition, Liu et al. reported that the
p53 expression in U251 glioma cells was up-regulated by curcumin, and the G2/M cell cycle arrest was also induced by curcumin in a dose-dependent manner [65]. In terms of osteosarcoma, many studies have demonstrated the cytotoxicity of curcumin against various osteosarcoma cell lines [66-69]. More recently, Collins et al. investigated the cancer inhibitory effects of both curcumin and garcinol, and showed that curcumin could induce the apoptosis of U2OS and SaOS2 osteosarcoma cell lines by up-regulating p53 expression [70].

2.5 Properties and Applications of Nanosale Self-assembled Amphiphilic Peptides

Nanomaterials are materials with at least one dimension from 1 to 100 nanometers. With the smaller size and greater surface energy, nanomaterials can be developed for various applications [71]. In terms of the delivery system for curcumin, various methods including nanoparticulate curcumin, liposomes, nanoemulsions, and polymeric micelles have been investigated [51]. The bottom-up approach is attractive for drug delivery, in which nanosized building blocks, such as polymers and magnetic particles, are used as drug carriers since they can assemble higher-ordered structures [72]. In the category of the bottom-up approach, spontaneous self-assembly has been investigated recently, and studies focusing on the self-assembly of amphiphiles may be a forerunner to the development of bottom-up nanotechnology since these molecules can mimic the functions of biological systems. In biological systems, many component molecules can exhibit functions depending on their integrated actions and assemblies, such as proteins, nucleic acids, and lipids [73, 74]. For example, phospholipids are the main constituents of
cell membrane bilayers. They contain both hydrophobic aliphatic chains and hydrophilic phosphoglycerides on the other end, and thus they can assemble into bilayers [12].

Amphiphilic molecules, such as lipids, surfactants and copolymers, have simple structures and have been used widely in various fields including detergents, pharmaceutical sciences, food industries, and in catalysis [73]. A few decades ago, peptide amphiphiles were developed as effective self-assembly nanomaterials since they have remarkable biocompatibility and simplicity [75]. Herein, the self-assembly properties of amphiphilies and their applications in drug delivery are discussed.

2.5.1 Mechanisms of Self-Assembly Properties

Amphiphilic molecules mostly consist of two or more distinct moieties with various polarities that interact differently with solvents. The tail group in each of the amphiphilic molecules has solvophobicity, whereas the head group has solvophilicity. For example, in most of the cases that water serves as solvent, the hydrophilic (water-loving) moiety can interact with water molecules by polarity. The polarity groups can be positively or negatively charged ionic groups, zwitterionic groups that contain both negative and positive charges, and nonionic neutral groups. On the other hands, the hydrophobic (water-hating) portion is usually insoluble in water since this portion always contains a hydrocarbon or fluorocarbon chain (i.e., an n-alkyl aliphatic group and perfluoroalkyl group) [73].

2.5.1.1 Hydrophobic Interactions

The hydrophobic effect is defined as the disaffinity of oil for water with unusual
temperature dependence. The hydrophobic effect is crucial in micelle formation, protein folding, chromatographic retention, microemulsions, and bilayer formation. The hydrophobic tails tend to avoid direct contact with water, forming oily interiors and watery exteriors [73, 76, 77]. The aggregates of hydrophobic molecules in water are nearly nonpolar particles and nearly spherical clusters acting like cavities, and the driving forces for this action are hydrophobic effects and weak attractive Van der Waals interactions that affect the position of an oil-water interface. When there are sufficient numbers of hydrophobic molecules clustered together, the total free energy $\Delta G$, which is given by the subtraction from the changes of enthalpy by the changes of entropy multiplied by temperature induced during solvation ($\Delta G = \Delta H - T \Delta S$), is lower than the free energy incurred by all the individual hydrophobic molecules. Since lower free energy is more favorable in a heterogeneous system, the aggregation of hydrophobic molecules in water is spontaneous. This tendency of aggregation can be the driving force of hydrophobic effects.

In terms of amphiphile assembly, with the addition of hydrophilic units conjugated with the hydrophobic groups, additional entropic effects arise because both hydrophobic and hydrophilic interactions have to be accommodated and the molecular configurations are restricted. Therefore, amphiphilic assembly can be formed, and micelle formation is a typical example (Figure 2.8) [77].

Also, Ramanathan et al. mentioned the entropic contribution on the formation of self-assembled aggregates of amphiphiles. The formation of hydrogen bonding among water molecules due to higher entropy can arise. With the hydrophobic tail groups of
amphiphilies, aggregated structures are entropically more favorable than the segregated amphiphilic molecules. This can result from the adsorption at the interfaces for avoiding hydrophobic groups from direct contact with water. Otherwise, the segregated amphiphilic molecules can interrupt hydrogen bonding, which is not entropically favorable [73].

![Figure 2.8 Amphiphiles in dynamic equilibrium with micelles. The blue spheres represent hydrophilic groups and red spheres represent hydrophobic groups. The radius of a micelle consisting of n molecules is given by $L = (\alpha^2 \delta^{1/3}) n^{1/3}$, where $\alpha$ and $\delta$ is the typical width and length of a single molecule (adopted from [77]).](image)

2.5.1.2 Electrostatics

Some amphiphilic molecules contain charged groups in the hydrophilic portion that can directly interact with water molecules by polarity. In these charged molecules, electrostatic forces can simultaneously offer attractive and repulsive intermolecular forces that control the dynamics of self-assembly. The energy representing the electrostatic interaction between two molecules is proportional to the number of charges but inversely proportional to the intermolecular distance [74].

Many studies have demonstrated the importance of electrostatic interactions on the morphologies of self-assemblies [79-81]. Jin et al. investigated the self-assembled
morphismes of amphiphilic peptide derivatives with different peptide sequences that exhibit different electrostatic interactions. In their study, the same aliphatic tail group was conjugated with two different amino acid sequences. One of these sequences contained valine (V)-arginine (R)-glycine (G)-aspartic acid (D)-valine (V), while valines were replaced by glutamic acid (E) and had higher anionic charges on the other one. The morphologies were characterized by transmission electron microscopy (TEM). Their observations showed that amphiphilic peptides with valines formed nanofibers, whereas peptides with higher anionic charges formed spherical micelles when pH=7 (Figure 2.9). These results may show that the repulsion forces induced by higher electrostatic interactions can alter self-assembly morphologies [79].

Figure 2.9 TEM images of self-assembled nanofibers formed by peptide sequence with lower electrostatic interactions (left) and spherical micelles with higher electrostatic interactions (right) (adopted from [79]).
2.5.1.3 Hydrogen Bonds

Hydrogen bonds are intermolecular interactions between polar molecules, in which hydrogen atoms are attracted to an electronegative atom including oxygen, nitrogen and fluorine. Hydrogen bonds can be considered as one type of electrostatic interaction since the hydrogen atom has a partially positive charge when covalently bonded to an electronegative atom, and the attraction is then caused by a nearby partially negative charge of another electronegative atom [74].

Hydrogen bonds can allow for the formation of well-defined β-sheet helical structures and higher-ordered architectures [82]. Under appropriate chemical- or medium-induced triggering, hydrogen bonds can lead to well-ordered and three-dimensional tubular structures, constructing stable self-assembled nanotubes that have variable inner channels and outer surfaces depending on the choice of functional hydrophilic groups [83]. Fenniri et al. designed a novel rosette nanotube in which hydrogen bonds served as the driving force of the self-assembly structure (Figure 2.10). The rosette nanotube is composed of building blocks that possess the Watson-Crick donor-donor-acceptor (DDA) H-bond array of guanine and acceptor-acceptor-donor (AAD) of cytosine. In physiological conditions, six of these building blocks could form a supermarcocycle by 18 hydrogen bonds. The tubular structure was organized by energetically favorable stacking effects and hydrophobic interactions. The self-assembled rosette nanotube is a hollow inner channel with diameter of 11Å and outer diameter of 3.5 nm [84-86].
Critical Micelle Concentration

In solution, aggregates of amphiphiles can behave differently. The structures can be spherical, rod-like, bilayers, globular, and even donut-like [75]. As discussed earlier, micelles are important structures since many types of amphiphiles can associate into the micelle structure. Unlike spherical bilayers that contain an encapsulated aqueous phase inside the hydrophobic core, micelles have closed hydrophobic interiors.

Micelles are formed at a certain concentration of amphiphilics. This concentration is called the critical micelle concentration (CMC) [73-75, 77]. In dilute solutions with concentrations lower than the CMC, micelle aggregates cannot be built up in stable three-dimensional structures [87].

For concentrations lower than the CMC, the physicochemical properties (i.e., surface tension and diffusivity) change gradually with the surfactant concentration. In contrast, the physicochemical properties change sharply at the critical micelle concentration (Figure 2.11) [73, 88].
The CMC is affected by hydrophobic interactions induced by the hydrophobic tail group. Chen et al. synthesized a surfactant-like tertra-tail amphiphilic peptide, and found that the addition of four fatty tails enabled a lower CMC and more stable micelles through strong hydrophobic interactions [89].

Figure 2.11. Changes of physical properties at the critical micelle concentration [73]

2.5.3 Solubilization In Self-Assembled Micelles

The low solubility in water of many types of anti-cancer drug limits their therapeutic efficacy. This inefficiency can be rendered by the direct exposure to harsh biological environments, uncontrolled binding, aggregation, and precipitation induced by poor solubility.

By encapsulating the hydrophobic molecules in the inner hydrophobic cores, micelles can be designed not only to entrap and solubilize the water-insoluble molecules, but also release the drug in a controllable and sustained manner in aqueous solution [90]. A number of studies have used self-assembled micelles as drug delivery vehicles for hydrophobic molecules.

The encapsulation capability of micelles is always expressed by two parameters:
encapsulation efficiency (EE) and loading level (LL). The encapsulation efficiency is a ratio of the weight of hydrophobic drugs in micelles to the weight of the initial drug, while the loading level is the ratio of weight of drugs in micelles to the weight of micelles [73, 89, 93].

Yu et al. successfully prepared methoxy poly(ethylene glycol)-cholesterol micelles (PM) to deliver docetaxel (DTX), a hydrophobic anti-tumor drug, in aqueous solution. These micelles with the optimal formulation had a high EE of 97.6%, and diameter around 13.76 nm as characterized by TEM. The drug release profile of DTX from DTX-PM showed a sustained release of drugs in a pH-sensitive manner [89, 91-93].

2.5.4 Geometry of Self-Assembled Amphiphilic Aggregates

The formation of self-assembled amphiphilic aggregates can be regarded as a two-step process. In the first step, the curvature of the hydrophobic-hydrophilic film in the interface is formed, and the molecular architecture of amphiphilic molecules is formed in the second step.

Firstly, the geometry of the curvature of the amphiphilic film in the interface is determined by its mean curvature $H$ and Gaussian curvature $K$ as follows:

$$H = 0.5 \left( \frac{1}{R_1} + \frac{1}{R_2} \right)$$

$$K = \frac{1}{R_1 R_2}$$

$R_1$, $R_2$=two radii of curvature (Figure 12)

The geometry of the molecular architect of amphiphilic aggregate can be determined by the critical packing parameter (cpp), which is,
cpp=$v/\text{al}$

$v$=molecular volume

$a$=the effective area of the hydrophilic head group

$l$=the length of hydrophobic tail (Figure 2.12)

**Figure 2.12** Description of parameters that determine the mean curvature H, Gaussian curvature K, and critical packing parameter (adopted from [94]).

The effective area of the hydrophilic head group, $a$, is induced by the balance between the tendency of the hydrophilic head group to maximize their interaction with water and the hydrophobic effects of the tail group to avoid direct contact with water.

With the critical packing parameter, the shapes of the self-assembled aggregates can be determined (Figure 13). For spherical micelles, the cpp value is less than 1/3, and the increase of cpp can induce various shapes of aggregates from a cylinder to reverse spheres [73, 94].

### 2.5.5 Functionalized Surfactant-like Amphiphilic Peptides

Natural L-amino acids have excellent biocompatibility and have been investigated for self-assembled nanomaterial applications. The surfactant-like amphiphilic peptides have been developed by many investigators to mimic the structure of traditional
surfactants in the drug delivery field [95-97], tissue engineering [98-99], and gene delivery [100-101]. The surfactant-like amphiphilic peptides also contain hydrophilic parts composed of several hydrophilic amino acids and hydrophobic parts composed of aliphatic groups or hydrophobic amino acids. The hydrophilic amino acids used to design the surfactant-like amphiphilic peptides could be positively charged (such as arginine and lysine), or could be negatively charged (such as aspartic acid and glutamic acid). In terms of the drug delivery application of amphiphilic peptides, the surfactant-like amphiphilic peptides can potentially encapsulate hydrophobic drugs due to a hydrophobic core, and the hydrophilic head group can be functionalized for cell-targeting [74, 102, 103]. Here, two types of functionalized peptide sequences in the hydrophilic head group of amphiphilic peptides are introduced.

![Different shapes of amphiphilic aggregates depending on the critical packing parameter (adopted from [73]).](image)
2.5.5.1 RGD Targeting Peptide Sequence Against Overexpressed αvβ3 Integrin On Osteosarcoma Cells

Compared to normal cells, tumor cells feature an abnormal and uncontrollable cell cycle, cell proliferation, angiogenesis, cell survival and cell migration. Integrins are cell receptors recognizing extracellular matrix (ECM) proteins, which play an important role in angiogenesis and cell migration. In the growth of solid tumors, new vessels are established and tumor metastasis promotes tumor spreading to local and distant tissues from primary tumor sites, in which increased levels of expression of the αvβ3 integrin is observed for many cancer cells, including melanoma, glioma, and breast cancer cells [104, 105]. Recently, Hassenpflug et al. found that the metastatic behavior of osteosarcoma was associated with overexpressed αvβ3 integrins [106], and Toma et al. demonstrated that increased expression of αvβ3 integrin was observed in highly metastatic osteosarcoma in nude mice, indicating that this integrin could be crucial for osteosarcoma metastasis in vivo [107].

The overexpressed αvβ3 integrin might be a potential therapeutic target for the treatment against osteosarcoma [104-108]. Some “homing” peptide sequences are able to bind to the surface of tumor cells specifically and are smaller than antibody fragments. The Arg-Gly-Asp (RGD) tripeptide is an example of these homing peptide sequences. This tripeptide is able to bind to its receptor αvβ3 integrin present in extracellular matrix components including vitronectin, fibronectin, fibrinogen, lamin, collagen and others [106, 109].

Many studies have used micelles with RGD functionalized hydrophilic head
groups for targeted drug delivery. Raj et al. conjugated the RGD peptide sequence to a C16 or a C18 fatty-acid chain and 8-amino-3, 6 dioxaoctonoic acid (ADA) to form self-assembled micelles in aqueous solution. These micelles exhibited good stability and were not disrupted upon a 10-fold dilution by water. More importantly, after the anti-cancer drug paclitaxel was encapsulated in the micelles, higher selective cytotoxicity against A2058 melanoma cells that overexpressed the αvβ3 integrin was observed for the RGD-functionalized carriers [110, 111].

2.5.5.2 Arginine-Rich Cell Penetrating Peptides

In order to overcome the limits of the therapeutic application of biopharmaceuticals aroused by cell surfaces, various types of cell penetrating peptides (CPPs) have been developed to facilitate the intracellular delivery of membrane-impermeable bioactive drugs. The CPPs are short cationic and amphiphilic peptides and their derivatives, commonly consisting of arginine and lysine residues. In general, the cationic CPPs can be internalized into the cell membrane by direct penetration. A local invagination of CPPs on the cell membrane can be caused by the negative charge on the lipid bilayer and by the positive charge of the CPPs.

Endocytosis may also facilitate cellular uptake of CPPs. For most of the cell types except for some special cells involved in the immune system, pinocytosis plays an important role in taking up extracellular components. Pinocytosis consists of macropinocytosis, lathrin-mediated endocytosis, caveolin-mediated endocytosis, and one or more clathrin- and caveolin-independent forms [112].
Tat polypeptide (GRKKRRQRRRPQ) was first found from HIV-1 virus and studied as a non-viral CPP with low toxicity. In addition, arginine-rich CPPs are peptide sequences that contain consecutive 5 to 11 arginine residues. They were showed to internalize into cell membrane more efficiently than Tat peptide since the guanidinium residues of arginine-rich peptide can form bidentate hydrogen bond with cell membrane components (i.e., phosphate, sulfate, and carboxylate) [101, 127]. They are of growing interest since they can enhance the efficiency in penetrating the cell membrane without inducing notable toxicity. Cells can take up the cargo-carrying arginine-rich CPPs on their surface, and the bioactive cargo can be released in the cytosol or nucleus due to endosomal escape. The cellular uptake efficiency of arginine-rich CPPs is dependent on the number of arginine residues in the molecule. For example, octaarginine (R8) can exhibit efficient internalization and nuclear localization, whereas R16 has less internalization efficiency and R4 has no significant effects of internalization [113-116].

Macropinocytosis is hypothesized to be one of the major pathways for the cellular uptake of arginine-rich CPPs. For arginine-rich CPPs, the uptake is less dependent on heparan sulfate proteoglycans (HSPG), and a different mechanism exists for the internalization of CPPs different structures. Moreover, the internalization of R8 CPPs could induce significant rearrangement of the actin cytoskeleton even if the plasma member could remain intact [117-118]. Nakase et al. found that the interaction of arginine-rich peptides with proteoglycans on cell surface could trigger the increase activation of Rac protein, leading to the F-actin organization and the promotion of membrane ruffles. The macropinocytosis is then driven by the formation of membrane ruffles.
protrusion, and the extracellular substance such as arginine-rich peptides can be engulfed as macropinosomes (Figure 2.14) [128].

![Figure 2.14 Macropinocytosis-mediated endocytosis induced by arginine-rich peptides (adapted from [128])](image)

**2.6 Summary of Literature Review**

The development for safer and more targeted therapies against osteosarcoma is necessary to improve the overall survival rate of patients [1, 5, 6]. The factors of the occurrence of osteosarcoma include mutation of p53 and retinoblastoma (Rb) tumor suppressor genes, overexpression of the activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB) transcription factors, as well as chromosomal aberrations [7, 8, 12, 13, 16]. Unlike many chemotherapeutic agents that induce significant toxicity [24-48], curcumin, a natural polyphenol compound extracted from the root of Curcuma longa, has been shown to have anti-cancer effects [49] and little adverse effects [50]. Curcumin can inhibit the cell proliferation and survival of cancer cells by various molecular and cellular pathways. By serving as a free radical scavenger, curcumin can inhibit the activation of
NF-kB and AP-1, causing the cell cycle arrest of cancer cells at the G1/S phase [58-63]. Moreover, curcumin can also induce the intrinsic apoptotic pathway in cancer cells by the activation of p53 tumor suppressor gene [65-70]. However, the bioavailability of curcumin is restricted by its low solubility in water as well as high degradation rate in alkaline conditions [49, 53].

Bottom-up nanotechnology using self-assembled amphiphilic peptides are amphiphilies that contain natural L-amino acids. In addition to better biocompatibility, amphiphilic peptides can also be functionalized by a variety of peptide sequences for different applications [74]. For instance, the RGD tripeptide can target to the overexpressed receptors, ανβ3 integrin, on cancer cells [110-111, 124], while the cationic peptides with consecutive 5 to 11 arginine residues can facilitate the macropinocytosis-mediated pathway for cellular uptake [117-118, 122-123, 128]. Therefore, to design a surfactant-like drug carrier that can
deliver curcumin selectively and efficiently to cancer cells, an amphiphilic peptide with Arg-Gly-Asp (RGD) and arginine-rich sequence conjugating with fatty acid tail group was used in this study to selectively treat osteosarcoma cells.
**3.0 EXPERIMENTAL**

3.1 Materials

The cell culture medium Eagle's Minimum Essential Medium (EMEM) was purchased from ATCC, VA, USA, and the osteoblast basal medium and the osteoblast growth medium Supplement Mix were purchased from PromoCell, Heidelberg, Germany. Curcumin (diferuloylmethane), acetic acid, dimethyl sulfoxide (DMSO), 4',6-diamidino-2-phenylindole (DAPI) and Atto Rho6G phalloidin were supplied by Sigma Aldrich, MI, USA. The dry powder of the C18GR7RGDS (Figure 3.1) amphiphilic peptide was obtained from Biomatik, DE, USA. The PlusOne Mini Dialysis Kit (MWCO 1kDa) was purchased from GE Healthcare, Buckinghamshire, UK and the methyl thiazolyl tetrazolium (MTT) dye solution was purchased from Promega (Madison, WI, USA).
3.2 Preparation of Self-Assembled Amphiphilic Peptide Nanoparticles (APNPs)

The amphiphilic peptide nanoparticles (APNPs) were prepared by dissolving the dry powder of C18GR7RGDS (MW=1850.28 g/mole) in deionized (DI) water followed by sonication for 60s. In addition, the amphiphilic peptide was also suspended in phosphate buffer saline (PBS) and an acetic acid solution at pH=2, 4, and 6. Then the self-assembled behaviors of APNPs in different solutions were observed by TEM as described in the next section.

3.3 Transmission Electron Microscopy (TEM)

The morphologies of the APNPs in different solutions were determined by a JEOL, JEM-1010 transmission electron microscopy (TEM). Samples in different aqueous conditions were prepared by dissolving the amphiphilic peptides in DI water, PBS, and acetic acid solution at pH=2, 4, and 6. 5 µL of each sample was mounted on a 300-mesh copper grid (EM sciences), and then negatively stained by 5µL of 1.5% aqueous Figure 3.1 (i) Chemical structure of the C18GR7RGDS amphiphilic peptide. Region (a) hydrophobic fatty acid moiety; (b) Arginine-rich sequence; and (c) RGDS sequence; and (ii) three-dimensional schematic structure of the C18GR7RGDS peptides obtained by ChemBio3D Ultra 14
phosphotungstic acid for 5s. The excess liquid was removed carefully by filter paper. The images were captured by TEM at 40,000-50,000X operating at accelerating voltage of 80kV.

3.4 Preparation of Curcumin-Loaded Amphiphilic Peptide Nanoparticles

The curcumin-loaded APNPs were prepared by co-dissolution and dialysis tubing methods [119]. Firstly, curcumin was dissolved in 50% acetic acid and then co-dissolved with APNPs. In the mixture, the molar ratio of peptide to curcumin was equal to 1:2. The mixture was then transferred to a dialysis tube (MWCO 1kDa) against 800 ml of deionized water. The water was replaced by fresh deionized water every 4 hours in order to eliminate acetic acid and unloaded curcumin from the mixture in the dialysis tube. When the pH of the mixture was close to 7.0, the dialysis tubing was terminated. The morphologies of curcumin-loaded APNPs in the final solution were characterized by TEM as previously described.

3.5 Zeta (ξ)-potential Measurements

Zeta (ξ)-potentials of pure APNPs and curcumin-loaded APNPs were determined using a Malvern Nanosizer (ZS90, UK). 0.4 mg/ml of pure APNPs and curcumin-loaded APNPs were prepared in DI water followed by sonication for 60s at room temperature. Then, the zeta-potential of 1 ml of each sample was measured over 10 runs.

3.6 Calculation of Encapsulation Efficiency and Loading Level

The amount of curcumin encapsulated in the APNPs was characterized by a
standard curve showing a linear correlation between the known concentrations of curcumin in DMSO and the corresponding absorbance measured by Ultraviolet–visible (UV-Vis) spectroscopy (SpectraMax M3, Molecular Devices, CA, USA) at 430 nm wavelength ($R^2 > 0.98$). Briefly, an aliquot of the curcumin-loaded APNP solution was lyophilized by a freeze-dryer (FreeZone 2.5 Plus, LABCONCO, MO, USA). Then, the dry powder was dissolved in DMSO, and the concentration of curcumin was evaluated by correlating the UV-Vis absorption of this solution at a 430 nm wavelength to a concentration value by the standard curve. The concentration of curcumin was evaluated three times for each sample, and the average value was collected to evaluate the encapsulation efficiency (EE%) and loading level (LL%), which can be calculated by the following equations:

$$EE\% = \frac{\text{Weight of drug encapsulated}}{\text{Weight of drug added}} \times 100\%$$

$$LL\% = \frac{\text{Weight of drug encapsulated}}{\text{Weight of micelles}} \times 100\%$$

3.7 Fourier Transform Infrared (FT-IR) Spectral Study

The FT-IR spectra of plain curcumin, the C18GR7RGDS peptide powder, and the lyophilized curcumin-loaded APNPs were collected in order to analyze the chemical structure of these compounds and the possible changes of the chemical structure after drug loading. Samples were analyzed by a FT-IR spectrometer (Vertex 70, Bruker) using attenuated total reflectance (ATR) method. The FT-IR spectra were collected in the wavelength range from 550 to 4000 cm$^{-1}$ with a resolution of 2 cm$^{-1}$. 

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3.8 X-Ray Diffraction (XRD) Study

X-ray diffraction (XRD) study was conducted to analyze the crystallographic structure of curcumin, pure APNPs, and lyophilized curcumin-loaded APNPs. Samples were analyzed by X-ray diffractometer (Ultima IV, Rigaku, Japan) at a voltage of 40 kV, 44 mA and 1.76 KW. The scanned angle was in the range of $5^\circ \leq 2\theta \leq 40^\circ$ and the scan rate was $3^\circ$/min.

3.9 Cell Culture

MG-63 osteosarcoma (OS, CRL-1427, ATCC, USA) cells were cultured in Eagle’s Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, while the human osteoblast (HOB, C-12760, PromoCell, Germany) were cultured in the completed growth medium composed of osteoblast basal medium and osteoblast growth medium Supplement Mix. Both cell lines were incubated in 37°C in a humidified incubator in an atmosphere of 95% oxygen and 5% carbon dioxide.

3.10 In Vitro Cytotoxicity Assay

MG-63 osteosarcoma (OS) and human osteoblast (HOB) cell lines were used to evaluate the cytotoxicity of plain curcumin suspended in PBS, curcumin dissolved in DMSO, a solution of pure APNPs, and a curcumin-loaded APNPs solution by a methyl thiazolyl tetrazolium-based colorimetric (MTT) assay. Initially, 100 µl of the cell solution of both OS and HOB cell lines were seeded on a 96-well plate at $2 \times 10^4$ cells/ml (6154
cells/cm²) cell density. After 24 hours of incubation in 5% CO₂ and 37°C for attachment, cells were treated by plain curcumin in PBS, curcumin dissolved in DMSO, and curcumin-loaded APNPs solution at different curcumin concentrations (3, 5, 10, 20, and 30 µM). For the cells treated with a solution of pure APNPs, the solution was prepared by the same co-dissolution and dialysis tubing method as that used for preparation of curcumin-loaded APNPs mentioned previously. The positive control samples were cells treated with cell medium only. For the samples treated with curcumin dissolved in DMSO, the same amount of DMSO (less than 0.5%) was used to treat the cells as control samples. Serum-free mediums were used in all of these samples to avoid the interaction of arginine-rich peptides on serum proteins.

Cells were then treated for 24 hours. After this, the medium from each sample was removed and cells were washed three times with PBS. Followed by the addition of 100 µl of cell medium, 15 µl of the MTT dye solution was added to each well, and the cells were incubated for 4 hours to allow for the formation of formazan crystals. At the end of incubation, 100 µl of the stop solution of MTT was added to each well. The 96-well plates were then tested using a spectrophotometer (SpectraMax M3, Molecular Devices, USA) at a 570 nm wavelength to obtain the optical density. Cell density was obtained from a standard curve expressing the linear correlation between different cell densities and optical densities ($R^2 > 0.98$). The cell viability was expressed as the ratio of cell density in each sample to the cell density in the control sample.
3.11 Qualitative Cellular Uptake Study

For the qualitative cellular uptake study, a confocal laser scanning microscopy and a bright field microscopy were used. 1ml of both OS and HOB cell lines were seeded on a 24-well plate at a seeding density of $2 \times 10^4$ cells/ml. After 24 hours of incubation in 5% CO$_2$ and at 37°C, cells were treated for 2 h with 20 µM of curcumin encapsulated in APNPs and plain curcumin suspended in PBS. The cells were then rinsed with PBS three times to remove the unabsorbed curcumin. The qualitative uptake of curcumin was then monitored by bright field microscopy.

For observation by confocal laser scanning microscopy (LSM710, ZEISS, USA), after 10 min of fixation by a 10% formaldehyde solution and subsequent treatment with a 0.1% Triton X-100 solution for another 10 min, the nucleus of the cells were stained by DAPI and f-actin were stained by Atto Rho6G phalloidin. The stained cells were then viewed under a confocal microscope for DAPI (excitation at 358 nm, emission at 461 nm), Atto Rho6G phalloidin (excitation at 525nm, emission at 560 nm), and curcumin uptake was observed using a FITC filter (excitation at 495, emission at 519 nm).

3.12 Statistical Analysis

Each experiment for each cell line was conducted at least three times, at five samples for each group. Data are expressed as mean ± standard error of the mean and a two-tailed Student t-test was used to evaluate differences between means, with P<0.05 being considered statistically significant.
4.0 RESULTS AND DISCUSSION

4.1 Characterization of the Self-Assembly Behavior of APNPs

This study showed that the C18GR7RGDS amphiphilic peptide could be dissolved in deionized water, PBS (pH=7.4), and acetic acid (pH=2, 4, and 6) easily. Initially, a stock solution of 10 mg/ml of APNPs in autoclaved deionized water was prepared, and then preserved at -80°C before use. In order to estimate the minimum concentration at which the amphiphilic peptide could self-assemble, three fixed concentrations (0.5, 0.75, and 1.5 mg/ml) of peptides in deionized water were investigated first by TEM. The morphology of the self-assembled C18GR7RGDS amphiphilic peptide nanoparticles (APNPs) in different solutions was also characterized by TEM.

In deionized water and PBS, the TEM images showed that the peptide could self-assemble into nanospheres with diameters of 10-20 nm at all the concentrations investigated (Figure 4.1 (a)-(c), (e)). The C18 aliphatic tail group could serve as the driving force of the self-assembly behavior of APNPs. The hydrophilic head group of the peptide functionalized by positively charged arginine-rich could result in strong electrostatic interaction between each adjacent molecule. A spherical morphology of APNPs was, thus, formed by the equilibrium of the hydrophobic interaction led by the tail group and the electrostatic interaction led by the head group [76-78]. Interestingly, it was found that the APNPs could aggregate (Figure 4.1 (d)) with each other when the peptide was dissolved in deionized water without sonication before characterization.
In an acetic acid solution, the self-assembled nanospherical aggregates could still be observed at pH=6 (Figure 4.1 (f)). However, at a pH=2 and 4 (Figure 4.1 (g)-(h)), only random cloud-like layers were observed, and the amphiphilic peptides could not self-assemble into nanospheres. Therefore, APNPs could be opened in acidic conditions at pH values less than 4, and allow for external material encapsulation as the self-assembly behavior could be pH-sensitive. Since curcumin is soluble in acetic acid, it is possible to encapsulate curcumin into APNPs aggregates by co-dissolution with acetic acid to disrupt the self-assembled structure, and to reform the nanoparticles by removing acetic acid using a dialysis-tubing purification method. Arginine deprotonation may not be the main factor of this pH-sensitive property since the pKa value of arginine is 12.48, which denotes that the guanidinium groups on the arginine-rich structure could be positively charged in a physiological environment. The possible reason for the dissociation of APNPs in low pH might be that the electrostatic interaction is so strong that the repulsive force among molecules can disrupt the self-assembled structure.

This pH-sensitive property might benefit for the cellular uptake of encapsulated bioactive molecules in the inner core. For example, endosomes, in which the pH is from 5 to 6, are membrane-bounded compartments that can transport extracellular molecules from the plasma membrane to the lysosome. The lysosomes can engulf the molecules by digestive enzymes at a working pH around 4 to 5. Therefore, this low pH environment could cause the dissociation of micelles and the release of bioactive molecules to cytosol followed by the digestion by the lysosome.
Figure 4.1 TEM images of APNPs (as pointed by the arrows) at different concentrations: (a) 0.5 mg/ml; (b) 0.75 mg/ml; (c) 1.5 mg/ml; (d) Aggregates of APNPs without sonication at a concentration of 1.5 mg/ml; (e) APNPs in PBS (pH=7.4); and (f) acetic acid at pH=6, (g) disrupted APNPs in acetic acid at pH=4, and (h) pH=2. Images were taken at 40,000-50,000X magnifications.
4.2 Formation of Curcumin-Loaded APNPs

As APNPs could be disrupted by acetic acid when the pH is less than 4, here a method of co-dissolution with curcumin in 50% acetic acid was developed to open up the aggregates. Due to their low molecular weights, the acetic acid and the free curcumin molecules could be purified with dialysis tubing. Only APNPs and the encapsulated curcumin could be retained in the solution. By this method, a homogeneous solution of curcumin was successfully prepared with the help of APNPs. Compared to the same amount of a solid curcumin suspension in water (solubility less than 0.1mg/ml), the resulting solution (EE%= 8.4±2.5%, LL%=3.6±1.2%) showed significantly increased solubility and homogeneity (Figure 4.2 (a)). Moreover, this solution exhibited stability even after lyophilization. The lyophilized powder of the solution could be re-dissolved in water easily. Thus, we could hypothesize that APNPs could increase the solubility of curcumin in aqueous solution (Figure 4.2 (b)-(c)).

Subsequently, nanospheres were observed in the TEM images of the resulting solution after drug loading. These nanoparticles had similar morphology as the pure APNPs but with comparably larger diameters around 18-30 nm (Figure 4.3). This result could indicate that the behavior of self-assembly was not altered during the drug preparation procedures, and the nanoparticles were able to reform after the removal of acetic acid. Meanwhile, hydrophobic molecules, like curcumin here, could be entrapped and solubilized in the stearyl C18 aliphatic cores of the micelles by energetically favorable hydrophobic interactions [78, 90], suggesting successful drug encapsulation in the aqueous surfactant solution of APNPs.
The measured zeta-potential of pure APNPs was +60.53mV, while that of curcumin-loaded APNPs was +69.75mV. This result indicates that both pure and curcumin-loaded APNPs are stable in aqueous solution. After drug loading, the micelles
possessed higher zeta-potential, and the positively charged micelles could facilitate membrane potential-mediated cellular uptake [123].

**Figure 4.4 Zeta-potential of pure APNPs and curcumin-loaded APNPs**

4.4 Fourier Transform Infrared (FT-IR) Spectra Studies

To further investigate the chemical and molecular interactions after drug loading, FT-IR analysis was used to show the spectra of solid-state plain curcumin, pure APNPs, and curcumin-loaded APNPs (Figure 4.5).

In the spectra of plain curcumin, the bands that appeared in the ranges of 1225-1175 cm\(^{-1}\) and 1125-1090 cm\(^{-1}\) together with two additional weak bands in the ranges around 1070-1000 cm\(^{-1}\) could represent the 1:2:4-substitution of the aromatic rings. The two C=C bonds conjugated with the neighborhood aromatic rings and C=O bonds could be characterized at 1629 cm\(^{-1}\) and 1606 cm\(^{-1}\), respectively. The hydroxyl group with intramolecular hydrogen bonds in the phenol groups could be characterized by the relatively weak absorption at 3519 cm\(^{-1}\) [120].
In the spectra of pure APNPs, the absorption at 1654 cm\(^{-1}\) could represent the amide I group [119], while the band at 1560 cm\(^{-1}\) could indicate the COOH group in the amino acid sequence. In addition, the two wide bands at 3400-3300 cm\(^{-1}\) could characterize the amine group of the arginine-rich structure. For the spectra of lyophilized curcumin-loaded APNPs, the bands appeared in the similar wavelength as the pure APNPs, but the band at 1409 cm\(^{-1}\) could represent the OH deformation vibration on phenols [120]. The FT-IR spectra may suggest that the chemical structure of the amphiphilic peptide was not altered after drug loading since no significant band shift observed. Furthermore, most of the absorbance bands of curcumin could not be observed except for the OH deformation vibration on the phenols. This result could be an indication for the successful encapsulation of curcumin by APNPs, as curcumin molecules were shielded in the inner core of micelles, and the infrared radiation could not be transmitted through the encapsulated molecules.

4.5 X-Ray Diffraction (XRD) Study

X-ray diffraction (XRD) study was conducted to analyze the crystalline and molecular structures of curcumin before and after encapsulation by APNPs (Figure 4.6). In the XRD pattern of curcumin, a series of characteristic peaks can be observed in the range of 15°≤2θ°≤30°, which represents the distinct crystalline structure of molecules of curcumin. In contrast, the pure APNPs may not have characteristic crystalline structure since no evident peaks were observed in its XRD pattern. More importantly, the curcumin-loaded APNPs showed a similar XRD pattern to that of pure APNPs and did
not show an observable crystalline structure. The disappearance of characteristic peaks of curcumin crystalline structure can result from the encapsulation by APNPs. As the XRD pattern of the pure APNPs demonstrated that the molecules of APNPs could be in disordered crystalline structure or amorphous, APNPs could shield the curcumin molecules from diffracting the X-ray beams, causing the X-ray diffraction of curcumin crystalline structure undetectable. Thus, the XRD pattern of curcumin-loaded APNPs can further confirm the successful drug encapsulation.

Figure 4.5 FT-IR spectra of (i) solid-state plain curcumin, (ii) pure APNP, and (iii) curcumin-loaded APNP
4.6 In Vitro Cytotoxicity Assay

The cytotoxicity of the curcumin-loaded APNPs solution, the solution of pure APNPs, curcumin dissolved in the DMSO solution, and plain curcumin suspended in PBS was investigated. The pure APNPs showed minor cytotoxicity on both OS and HOB cell lines (Figure 4.7(a)), which might be caused by the increased positive charge along with the increased peptide concentrations [123]. The cytotoxicity of plain curcumin suspended in PBS was also insignificant for both of these two cell lines (Figure 4.7(b)-(c)), which might result from the low cellular uptake due to the low solubility of curcumin in PBS. On the other hand, the curcumin-loaded APNPs not only exhibited a concentration-dependent cytotoxicity, but also showed a significantly selective inhibition.

Figure 4.6 XRD patterns of curcumin-loaded APNPs, pure APNPs and curcumin
against the viability of OS compared with the curcumin/DMSO sample. At the curcumin concentration of 30 µM, the cell viability of OS was as low as 15% after treatment with curcumin-loaded APNPs, while over 50% of HOB were viable. The curcumin-loaded APNPs with 20 µM curcumin concentration could be the optimal concentration, in which the cell viability of OS had reached its minimum value. This result quantitatively confirmed the targeting effects of the RGD peptide sequence to the overexpressed αvβ5 integrins on cancer cells, thus, introducing higher cellular uptake of encapsulated drugs by OS than HOB.

Figure 4.7(a) Cytotoxicity of pure APNP to HOB and OS cells expressed by (i) cell viability and (ii) cell density. Data are expressed by S.E.M. and N=2 (5 samples per N). *P<0.01, **P<0.005.

Figure 4.7(b) Cytotoxicity of curcumin-loaded APNPs, plain curcumin in PBS and curcumin dissolved in DMSO to OS cells expressed by (i) cell viability and (ii) cell density. Data are expressed by S.E.M. and N=3 (5 samples per N). *P<0.01, **P<0.005.
4.7 Qualitative Cellular Uptake of Curcumin-Loaded APNPs

The internalization of curcumin-loaded APNPs by both OS and HOB cell lines was tracked via bright field microscopy and confocal laser scanning microscopy. After incubation with curcumin-loaded APNPs at a 20 µM curcumin concentration for 2 h, in the bright field microscopy images (Figure 4.6), OS cells showed a significantly higher cellular uptake of curcumin than the normal HOB cells. In the samples treated only with plain curcumin suspended in PBS, very small amounts of crystalline curcumin could be observed in the cell membranes, but curcumin did not accumulate in the cytosol by endocytosis and the internalization was inefficient without using the novel APNPs.

In confocal microscopy images (Figure 4.7), the nucleuses of the cells were tracked via a blue fluorescent stain DAPI, and the F-actin of cells were stained with the red fluorescent Rhodamine 6G. The cellular uptake of curcumin was monitored by a FITC filter (green fluorescent) [121,122]. Similar to the images taken by bright field microscopy, both cell lines did not show a detectable fluorescence of curcumin in the samples treated by plain curcumin, which indicated that APNPs could also enhance...
cellular uptake. In the samples treated by curcumin-loaded APNPs, OS cells showed a strong green fluorescent, indicating that cells accumulated significant amounts of curcumin into the cytosol. However, the normal HOB cells only showed a weak green fluorescent in their cytosol.

These results demonstrated that curcumin-loaded APNPs could penetrate the cell membrane of OS cell more efficiently and induce significantly higher cellular uptake than normal HOB cells. With an RGD functionalized head group, the curcumin-loaded micelles might selectively attach to the receptors of the overexpressed integrins on the OS cells, causing higher accumulation of the drug on the cell surface of OS cells than normal HOB cells. Meanwhile, the micelles could stably attach to carboxylate, sulfate, and phosphate groups on the cell membrane by two hydrogen bonds of each arginine group on the peptide sequence. This would favor the macropinocytosis-mediated internalization of arginine-rich peptides [123]. Hence, curcumin molecules could internalize into cytosol efficiently through the endosomal pathway from the cell membrane to lysosome, although the exact mechanisms of which will have to be elucidated in future studies.
Figure 4.8 Bright field microscopy images of normal human osteoblast cell (a) control, (b) treated with 20 µM of curcumin in PBS, and (c) treated with 20 µM curcumin encapsulated by APNPs; and osteosarcoma cell (d) controls, (e) treated with 20 µM of curcumin in PBS, and (f) treated with 20 µM of curcumin encapsulated in APNPs. Images were taken at a magnification of 20X.
Figure 4.9 Confocal microscopy for the cellular uptake of normal human osteoblast cells (a) controls, (b) treated with 20 μM of curcumin in PBS, (c) treated with 20 μM curcumin encapsulated by amphiphilic peptide nanoparticles; and osteosarcoma cell (d) controls, (e) treated with 20 μM of curcumin in PBS, and (f) treated with 20 μM curcumin encapsulated in APNPs. Images were taken at a magnification of 10X.
5.0 CONCLUSIONS

In conclusion, the present TEM results showed that the amphiphilic peptide C18GR7RGDS could self-assemble into spherical nanoparticles with diameters of 10-20 nm in water and PBS. This self-assembly behavior might be driven by the hydrophobic interaction of the aliphatic tail group and the electrostatic interaction attributed by the positive charge in the head group. The three-dimensional schematic molecular structure of the amphiphilic peptide was also obtained and analyzed by ChemBio3D Ultra 14 software (Figure 3.1(ii)). The estimated length of an amphiphilic peptide molecule is about 6.74 nm. From the comparison between the diameters of micelles measured in the TEM images and this schematic measurement, the micelle structures of APNPs could be monolayer aggregates with solid hydrophobic cores, although the critical packing parameter (cpp) should be precisely evaluated. Furthermore, since micelles could form when the concentration of amphiphilic molecules is above the critical micelle concentration (CMC) whereas the amphiphilic molecules behave differently when its concentration is lower than CMC [73], the morphologies of APNPs with lower peptide concentrations should be observed in order to study the stability of APNPs upon dilution.

The self-assembly property of APNPs were also found to be pH-sensitive. Under acidic conditions in which the pH was lower than 4, the aggregates of APNPs could dissociate and allow for the entry of external compounds. When the samples were negatively stained by uranyl acetate, the self-assembled structures of APNPs could not be observed by TEM (images not shown), which was possibly because the nanoparticles
were disrupted in the acidic uranyl acetate solution (pH=4.2-4.5).

By the method of co-dissolution with a curcumin solution in 50% acetic acid and dialysis tubing, APNPs could reform into spherical nanostructures but with greater diameters. More importantly, the solubility of curcumin significantly increased and the orange-yellow solution exhibited higher stability and homogeneity with the help of APNPs. Even after freeze-drying, the powder of the solute of this solution could easily dissolve in water instantly. Therefore, although the encapsulation efficiency and loading level were low, it can be hypothesized that curcumin could be successfully encapsulated in the inner hydrophobic core of the micelles of APNPs [78,90]. The encapsulation was further studied by the FT-IR and XRD analysis. As the result showed, after the drug loading process, no significant band shift in the FT-IR spectra of pure APNPs occurred, but bands representing most of the chemical groups in curcumin molecules could not be detected. This result might be evidence for the successful encapsulation of curcumin since the encapsulated molecules could be shielded from the infrared signal. The XRD analysis further confirmed the successful encapsulation of curcumin by APNPs since the distinct peaks for crystalline structure of curcumin disappeared and the curcumin-loaded APNPs had a similar XRD pattern as the pure APNPs did after drug loading.

In *vitro* cell experiments were conducted on both MG-63 osteosarcoma cells (OS) and normal human osteoblast (HOB) cells to investigate the targeting effect of APNPs against bone cancer cells. At curcumin concentration from 3 to 30 µM, the cytotoxicity of curcumin-loaded APNPs against OS cells was significantly greater than against HOB. The cell viability of HOB was 54% and OS was 15% after treatment with 30 µM of
APNPs with curcumin for 24 h, while the same amount of pure APNPs and plain curcumin suspension in PBS had low cytotoxicity on both cell lines. And 20 µM of curcumin concentration in APNPs could be the optimal concentration to inhibit osteosarcoma cell proliferation since the viability of OS had reached its minimum value. The selective cytotoxicity of curcumin-loaded APNPs was also greater than that of curcumin dissolved in the organic solvent DMSO, which could result from the RGD sequence targeting toward overexpressed integrins on cancerous cells [104-106, 124]. Since DMSO has been shown to able to enhance the penetration of both hydrophobic and hydrophilic agents into the cell membrane [129], curcumin could be deliver through the lipid membrane with the aid of DMSO. By incorporating below the hydrophilic head group in a non-specific manner, molecules of DMSO increase the average head group area and the amenability to bending in the lipid membranes, the entry of water molecules is then facilitated, inducing the water pore formation without interacting with any specific molecules [130]. However, although dermal application of DMSO did not show significant toxicity in some earlier studies, it was reported that DMSO had caused eye damages including lens abnormalities in animals [131]. Moreover, Hanslick et al. reported that DMSO could induce widespread apoptotic neurodegeneration in the central nervous system of juvenile mice, possibly leading to the similar damage in human children. Even at the concentration as low as 0.5%, DMSO could lead to the apoptosis in the central nervous system, and at the postnatal age of 7 and 30 that are equivalent to childhood ages of human, severe damages were observed after the exposure of DMSO [132]. Therefore, as one of the peaks of incidences of osteosarcoma in adolescents is in
10-14 year-olds when pubertal growth starts [6], DMSO might cause damages in the central nervous system when it serves as a solvent for hydrophobic anti-cancer drugs. In this study, APNPs did not show a significant toxicity, so the curcumin-loaded APNPs could excel curcumin dissolved in DMSO for the lower chronic toxicity induced by short polypeptides, although in vivo studies should be further investigated. Moreover, the drug delivery performances of APNPs should be compared with traditional drug carriers like liposomes.

In addition, the qualitative cellular uptake studies further confirmed the selectivity of curcumin-loaded APNPs. In both bright field microscopy and confocal laser scanning microscopy images, significant internalized APNPs with curcumin could be observed in the cytosol of OS cells, whereas little amount of curcumin was observed in HOB cells. Additionally, compared with cells treated with a plain curcumin suspension in PBS, the arginine-rich sequence of APNPs induced higher internalization efficiency, causing the macropinocytosis-mediated internalization of curcumin from the plasma membrane to the cytosol [123]. And since the self-assembled APNPs are pH-sensitive, the micelles of APNPs loaded with curcumin may be stable in early endosomes at pH=6, but disassemble in the lysosome at lower pH, releasing the drug by a controllable manner [95,96]. This assumption should be further confirmed by investigations on the cellular uptake mechanisms. In spite of this, it remains unclear if the APNPs could protect curcumin from degradation in alkaline conditions, so the stability of pure APNPs and curcumin-loaded APNPs in alkaline conditions should be studied. Also, in vivo studies are necessary to investigate the therapeutic efficacies of curcumin-loaded APNPs in the
In summary, the study demonstrated for the first time that the arginine-rich-RGD amphiphilic peptide could serve as a drug delivery vehicle for curcumin to selectively inhibit the proliferation of osteosarcoma cells. After loading with curcumin, the micelles of APNPs could induce selective inhibition against MG-63 osteosarcoma cells than normal human osteoblasts. Further studies for enhancing curcumin loading efficiency, stability of micelles, curcumin and gene combination delivery by APNPs, qPCR assays, and in vivo studies are necessary.
6.0 RECOMMENDATIONS

Although this thesis provides significant promising evidence for the application of APNPs to treat osteosarcoma, additional studies are necessary. The cationic peptides composed of arginine-rich sequences are good candidates for gene carriers due to their inherent bioavailability and biocompatibility. For example, APNPs could be used as a gene carrier combined with an encapsulated hydrophobic anti-cancer drug. Chen et al. had previously bound plasmid DNA on the surface of arginine-rich-RGD micelles. The studies conducted by Park et al. reported that amphiphilic peptides with arginine-rich sequences could induced higher transfection for plasmid DNA after being loaded with hydrophobic anti-cancer drugs such as curcumin and BCNU (Carmustine), indicating the potential of the application of the cationic peptides on drug and gene cancer combination therapy [124, 125]. Myc is a transcription factor that is overexpressed in bone marrow and essential to osteosarcoma development. The inactivation of Myc had been shown to induce cell cycle arrest of osteosarcoma [5]. Thus, the gene expressing Myc could be one of the targets of gene therapy against osteosarcoma.

Although curcumin-loaded APNPs might have a targeting effect against osteosarcoma cells and capability of facilitating cellular uptake, the mechanisms for these effects are still unclear. First, various functionalized amphiphilic peptides with or without RGD peptides should be investigated. A previous study replaced the RGD sequence with the GGG sequence, and thus confirmed the targeting effect of the RGD sequence to cancer cells with overexpressed receptors [110]. Secondly, the enhanced
endocytosis-mediated cellular internalization induced by the arginine-rich sequence should also be verified. Since lysine (K) is also a positively charged amino acid but composed of one hydrogen-bond donator, replacement of R with K amino acids could be used to investigate the importance of hydrogen bonds on cellular uptake efficiency. In addition, reduction of membrane potential using isotonic buffer could help analyze whether or not cellular uptake is driven by a membrane potential and is energy dependent [123, 126].

The need for enhancing encapsulation efficiency and loading level of APNPs should also be addressed. As these results showed, the encapsulation efficiency and loading level of APNPs were low, although the nanoparticles demonstrated a significant inhibitory effect against osteosarcoma cells even at this low level. The more strengthened hydrophobic interactions and more stable micelle formation could be achieved by conjugation with more fatty acid moieties in the hydrophobic tail group [89, 110, 119], and this should also be the focus of future studies. Moreover, in vivo studies could be carried out to further investigate the anti-cancer efficacies of curcumin-loaded APNPs.
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<th>Term</th>
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<tr>
<td>APNP</td>
<td>Amphiphilic peptide nanoparticle</td>
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<tr>
<td>Cpp</td>
<td>Critical packing parameter</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DI water</td>
<td>Deionized water</td>
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