PREPARATION OF CALCIUM CITRATE BASED VITAMIN B_{12}/CISPLATIN NANOCOMPOSITE AS NOVEL TARGETED DRUG CARRIER

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การเตรียมนาโนคอมพอสิตวิตามินบี12/ซิสพลาตินฐานแคลเซียมซิเทรตเพื่อเป็นตัวพายาสู่เป้าหมายชนิดใหม่

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Thesis Title	PREPARATION OF CALCIUM CITRATE BASED VITAMIN B_{12}/CISPLATIN NANOCOMPOSITE AS NOVEL TARGETED DRUG CARRIER
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Field of Study	Chemistry
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การสังเคราะห์นาโนคอมพอสิตซิสพลาตินแคลเซียมซิเทรต ถูกเตรียมผ่านการตกตะกอนร่วมทางเคมีอย่างง่าย หมู่คาร์บอกซิลบนผิวของอนุภาคนาโนแคลเซียมซิเทรตถูกดัดแปรผิวด้วยวิตามินบี 12 อนุภาคทรงกลมนาโน จากการสังเกตด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด อนุภาคที่ได้มีขนาด 553.0±65.0 นาโนเมตร การวิเคราะห์เปอร์เซ็นต์ของซิสพลาตินและวิตามินบี 12 อาศัยเทคนิคสมบัติทางความร้อน และ ICP-OES การตกแต่งภายในของอนุภาคนาโนถูกศึกษาด้วยเทคนิครามาน นอกจากนี้ การทดสอบความมีชีวิตด้วยเทคนิคเฟสโตบลูแสดงให้เห็นว่า นาโนคอมพอสิตวิตามินบี12/ซิสพลาตินฐานแคลเซียมซิเทรต ทำให้การรอดชีวิตของเซลล์มะเร็ง เอ-549 ลดลง 60 เปอร์เซ็นต์เมื่อเปรียบเทียบกับยาซิสพลาตินทั่วไป

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The synthesis of calcium citrate-cisplatin composite nanoparticles (CaCit@CDDP) was prepared via simple chemical co-precipitation method. Carboxyl group on the surface of calcium citrate nanoparticle was successfully modified with vitamin B12 (B12). The spherical nanoparticles were observed by Scanning Electron Microscope. The obtained size was 553.0±65.0 nm. The percentage of CDDP and B12 in the nanoparticles were analysed by Thermogravimetric Analysis and Inductively Coupled Plasma Optical Emission Spectroscopy. The interior of the nanoparticles was studied by Raman spectroscopy. Moreover, the Prestoblue viability assay showed that CaCit@CDDP-B12 significantly decreased 60% of cell viability of A549 cancer cell line in comparison to the conventional CDDP drug.

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Natchanon Rimsueb

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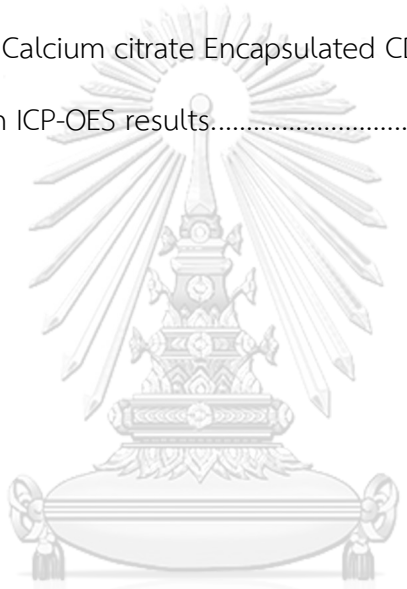

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CHAPTER I

INTRODUCTION

1.1 Background

In present, novel drug delivery systems can increase therapeutic efficacy and also reduce side effects of therapeutic agents by concentrating them at specific target sites in the body[1]. These systems can advantageously enhance therapeutic effectiveness by producing more favourable drug bioavailability, serum stability and pharmacokinetics. According to the literature, nano-controlled release formulations provide better penetration and allow slow and controlled release of active ingredients at a target site[2]. Many types of nano drug carrier were produced such as metal nano[3], polymer micelle[4], and ionic of calcium compound e.g. calcium carbonate ($CaCO_3$), calcium phosphate ($Ca(H_2PO_4)_2$) and hydroxy apatite ($Ca_{10}(PO_4)_6(OH)_2$)[5-7]. The advantages of $CaCO_3$ nanoparticles are biocompatibility, safety and easy to modify. Furthermore, the nanoparticles can control the release of drugs because of their longer biodegradation times.

Calcium citrate (CaCit) could be an alternative drug carrier. It is commonly used as a food additive, usually as a preservative. Additionally, calcium citrate is also found in some dietary calcium supplements (e.g. Citracal)[8]. In 2016, CaCit was prepared in nanosheet form to promote the formation of new bone. It implied that calcium citrate is also biocompatible, low cost and high clinical efficacy[9]. Recently, a CaCit microparticle has been synthesised as drug carrier[10]. Hydrophilic vancomycin (VAN) was encapsulated into CaCit which is used as antibiotic-impregnated PMMA bone-spacers in joint replacement surgery. However, there is no report that CaCit nanoparticles were synthesised *via* chemical precipitation for drug carrier.

Nanoparticles are widely used in cancer treatments as chemotherapeutic drug carrier. Various drug carriers have solved several limitations of conventional drug delivery systems such as non-specific biodistribution and targeting, lack of water solubility and poor oral bioavailability[11]. Moreover, the platinum-based drugs

cisplatin, *cis*-Diamminedichloroplatinum(II) (CDDP), has been widely used as a chemotherapeutic agent to treat a number of cancers. Additionally, cyanocobalamin or vitamin B12 (B12) can also enhance selectivity to a target cell *via* receptor-mediated endocytosis in the cancer cell[12]. Accordingly, this research focused on development of a novel CDDP encapsulated CaCit nanoparticles with surface modified by attaching vitamin B12 as a targeting probe.

1.2 Literature Reviews

1.2.1 Drug delivery system

Drug delivery has recently gained increasing interest as a method for administering pharmaceutical compounds to achieve therapeutic effects in humans or animals. The efficiency of the delivery process greatly depends on the nature of drug carriers, which are typically engineered to be within the nano-size range allow them to pass through semipermeable membranes[13, 14]. In particular, ionic calcium carbonate nanoparticles are one of the most commonly used drug carriers owning to their availability, low cost, safety, biocompatibility and slow biodegradability[1]. Calcium carbonate exists as three anhydrous crystalline polymorphs; rhomboidal calcite, needle-like aragonite and spherical vaterite. Calcite is the stable form, while aragonite and vaterite are the metastable forms that readily transform into the stable polymorph. Vaterite has the least stability and in contact with water it slowly dissolves and recrystallizes to calcite form. The different morphological forms of calcium carbonate related to the synthesis part for controlling size of the nanoparticles. There are many methods to prepare calcium carbonate nanoparticles such as reversed microemulsion method, double emulsion technique, O/W microemulsion method using a High-Pressure Homogenization (HPH), two-membrane dialysis system, chemical precipitation method. Preparation of drug-$CaCO_3$ nanoparticles has been generally synthesised by chemical precipitation[1]. The concept was to create an interaction between ion of calcium and carbonate in which two aqueous solution of separate ions are mixed together as shown in figure 1. Calcium chloride or calcium nitrate was usually used as calcium ion source and

carbonate ion source from sodium carbonate. Reaction between two aqueous phases leads to the formation of nanoparticles which can be separated through centrifugation. However, there are many factors to concern with particles size such as the speed of homogenization. The increase in speed leads to a greater mechanical shear which then result in a decrease in particle size. In addition, the ratio between calcium ion and carbonate ion will also affect the particle size because the rate of contact between two ions increases. Furthermore, some of the aqueous solution must firstly be interacted with drug before mixing the two solution together. By contrast, if they use a large amount of drug, the solution will decrease the viscosity rate of the solution which will decrease the shear stress and increase the contact rate of the ions and lead to an increase in particle size[15]. Decreasing the shear stress can also help to entrap more concentration of drug content because its increase the diffusion time of substrate.

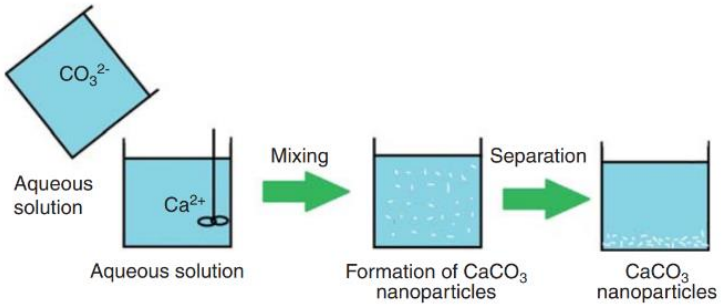


Figure 1. Chemical precipitation procedure for preparation of $CaCO_3$ nanoparticles.

In 2005, Ueno[16] and co-workers synthesised drug-incorporated calcium carbonate nanoparticles *via* simple chemical precipitation method. Betamethasone phosphate (BP) was used as drug encapsulated inside nanoparticles. The size of the nanoparticles was controlled by mixing speed. The reaction started with 650 µL of 5M $CaCl_2$ were mixed with 375 µL of 5% BP for 10 minutes. Next 2.5 mL of 1M Na_2CO_3 was added to the first aqueous solution and stirred vigorously for 10 minutes. After mixing, the solution was then added with 5 mL of distilled water and centrifuged at 2000 rpm for 5 minutes. The results show that the diameter of the

particles was dependent on mixing speed; gentle stirring at 650 rpm produced large particles, while vigorous stirring at 1300 rpm produced small particles. The range of distribution in particle size was narrower in the experiment. Furthermore, drug-incorporating calcium carbonate nanoparticles are successfully synthesised for a new delivery system

1.2.2 Importance of citrate ion

Due to their factors concerned with nanoparticle, this issue was overcome to some extent by using an enteric coating technique. Citrate ion is usually used as additives, stabilizing agents and also modification of the surface of the nanoparticles. In 2012, Ghiasi[17] and Malekzadeh synthesized calcium carbonate nanoparticles *via* so-called sol–gel citrate method using calcium nitrate as precursor in the presence of different concentration of citric acid. Citric acid was found to be a proper additive that decreases particle size of calcite on preparation. Citric acid is an important material to control a size of the nanoparticles. They found that the addition of citric acid, more than a half concentration of calcium nitrate, decreases the particle size of calcium carbonate about 50% on preparation. The morphology and crystallite size calculation are confirmed by the transmission electron microscopy measurement as shown in figure 2. Moreover, re-dispersing nanoparticles by sonication do not necessary for this preparation. It indicated that citric ions not only affect the size, but also stabilize the nanoparticles.

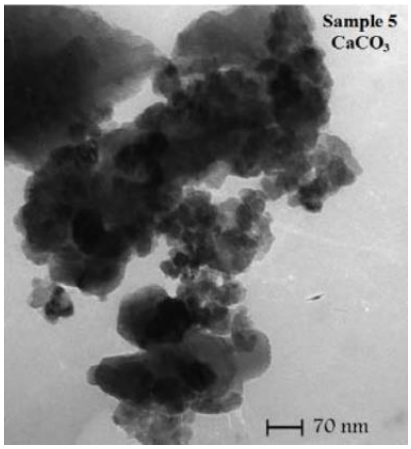


Figure 2. TEM micrographs of calcium carbonate.

In 2010, Leeuwenburgh[18] and co-workers developed polymer–ceramic composites for bone repair by using calcium phosphate nanoparticles. The main goal of this work is to study various dispersants which were evaluated for their potential to be used as biocompatible dispersants. Tribasic sodium citrate was selected as the most effective dispersant for the stabilization of calcium phosphate (CaP) suspensions. In the experiment, apatitic CaP nanoparticles were synthesised *via* chemical precipitation method involving the dropwise addition of ortho-phosphoric acid to an aqueous suspension of calcium hydroxide according to the following reaction (below).

$$5Ca(OH)_2 + 3H_3PO_4 \longrightarrow Ca_5(PO_4)_3(OH) + 9H_2O$$

The apatitic crystals were produced by slow dripping of 250 ml of a phosphoric acid solution (75 mM) to a basic suspension of 250 ml of $Ca(OH)_2$ (125 mM) at a rate of 3–4 ml min^{-1} and continuous stirring, yielding an apatite content of 0.625% (w/v). Then, the products were dispersed with sodium citrate tribasic dihydrate. Additionally, CaP powders in the presence of citrate were mixed with gelatin hydrogel to fabricated nanocomposite. Sedimentation experiments were figured out the effect of various dispersants on the stability of the CaP suspensions as shown in figure 3. The result indicated that tribasic sodium citrate was most effective in stabilizing CaP suspensions by adsorption of citrate ions onto CaP nanocrystals in CaP suspensions, thereby increasing the negative surface charge of the CaP particles and consequently increasing the repulsive interparticle forces. Consequently, aggregation and sedimentation of CaP mineral phase was reduced considerably.

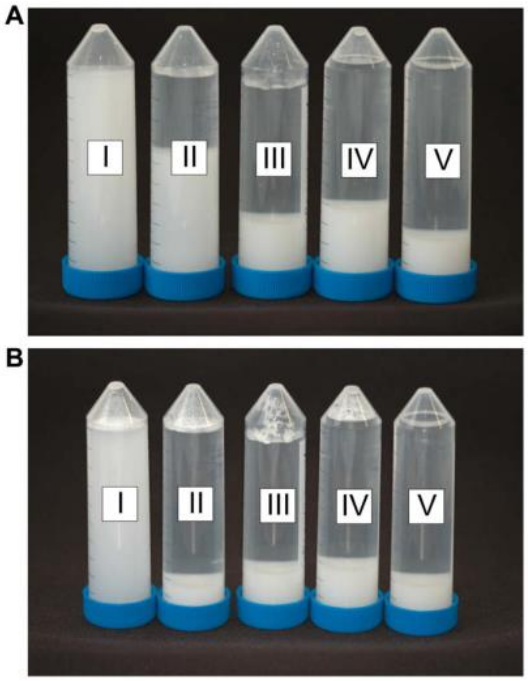


Figure 3. Sedimentation behavior after (A) 3 h and (B) 24 h of 0.625% (w/v) CaP suspensions supplemented with 0.1% (w/v) sodium citrate [I], citric acid [II], ammonium carbonate [III], Dolapix PC75 [IV], dispersant-free calcium phosphate suspension as a reference [V].

CaCit has been proved to be biocompatible as illustrated in the work reported by Li[9] and co-workers in 2016. CaCit nanosheets were prepared and used for promoting the formation of new bone in animal model. In this work, the calcium citrate nanosheets were able to control the release of calcium ions in high activity and high concentration during a short period of time, thus stimulating bone formation efficiently. The SEM micrographs (fig. 4) revealed the shape of the calcium citrate prepared at different ratio of alcohol: water. The sheet is thicker, relatively smooth and its edges and corners are not clear when the ratio of alcohol: water is 2:1. This observation could be explained by the lower crystallinity of the obtained calcium citrate. When the volume ratio is reduced to 1:2, the nano-calcium citrate formed a thinner sheet with a regular morphology and exhibits some hexagonal lamellar structures. However, calcium citrate particles had a tendency to agglomerate because the smaller thickness and larger width.

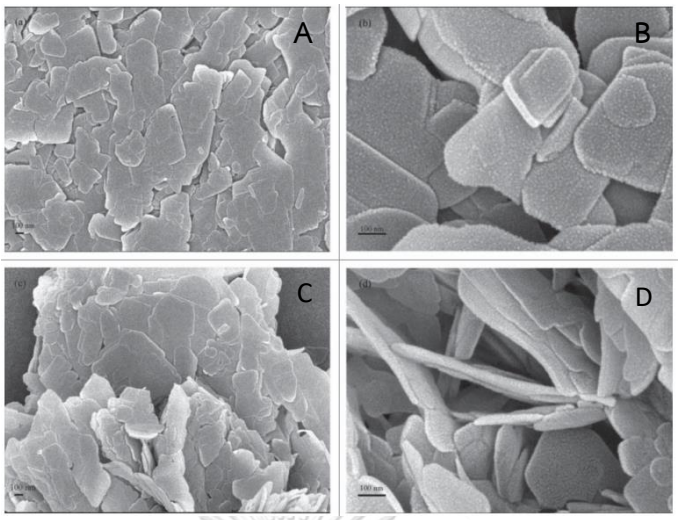


Figure 4. SEM micrographs of nano-calcium citrate powders produced at different ratio of alcohol: water: (A) alcohol: water=2:1, x30000 (B) alcohol: water=2:1, x100000 (C) alcohol: water=1:2, x30000 (D) alcohol: water=1:2, x100000.

Recently, Oungeun[10] and co-workers have successfully prepared CaCit particles encapsulated with vancomycin and incorporated into polymethyl methacrylate (PMMA) for making bone cement with prolonged drug release character in 2019. PMMA has been usually used as a bone-spacer to temporarily take up the space where the infected implant has been removed. Local delivering of antibiotics plays an important role of the material in addition to providing temporary structure and securing the space when PMMA were used as a bone-spacer. In this work, antibiotic-impregnated PMMA bone-spacers are usually made by a direct mixing of powdered antibiotics into the materials prior to the polymerization setting of the cement. Prolonging PMMA incorporated antibiotic drug should be concerned both degrees of cross-linking and homogeneity. Additionally, a surfactant has been used to help blend liquid antibiotics into the PMMA matrix. In this work, there are four different drug carriers to help in blending both hydrophilic and hydrophobic antibiotics into PMMA. One of these is calcium citrate particles and vancomycin (VAN) was used as antibiotic drug in this study.

In the synthesis parts, calcium citrate (CaCit) particles were prepared *via* chemical precipitation. The reaction was produced by mixing between $CaCl_2$ (Ca^{2+} source) and trisodium citrate (citrate source) with 1:1 mole ratio. In the process, 200 mg VAN and $CaCl_2$ were mixed together then added into trisodium citrate solution. The mixture was then continuously rocked (in an incubator shaker) at room temperature for 24 hours. After that, sodium hydroxide (3.31 mg) was added into the suspension to reduce the solubility of VAN and shaking was continued for another 24 hours. The resulting suspension was centrifuged at 9400 rpm for 30 min. VAN-loaded CaCit particles were collected by freeze-dried.

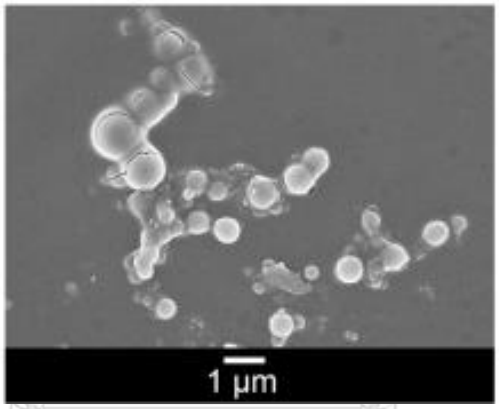


Figure 5. VAN-loaded CaCit particles.

The obtained size (fig. 5) is 554.4 ± 220.3 nm. CaCit particles were loaded with VAN only 4.9 ± 3.2% which is assembled from calcium and citrate ions. It was likely that ionic interactions between carboxylates in the VAN and Ca^{2+} took place. In addition, the phenolate could also form an ionic bond with the Ca^{2+}. The structure of VAN was shown in figure 6. Nevertheless, low encapsulation efficiency and low loading were observed because VAN probably could not very well compete with the much smaller citrate ions during the particle growth.

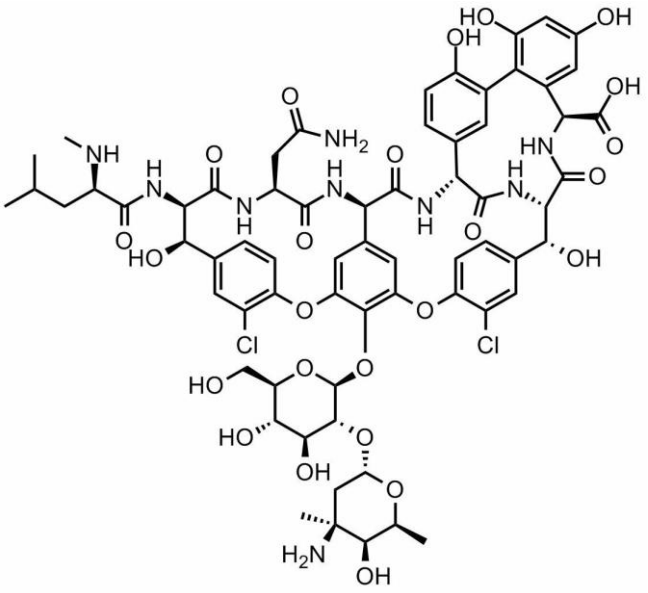


Figure 6. Vancomycin (VAN).

For application, the drug-incorporated PMMA cements were prepared. The significantly higher concentrations of the released drug were observed for the VAN–CC–PMMA likely caused by the presence of many micro/nanochannels in the composite matrix. Moreover, the compressive strengths are shown as the maximum stress values of the tested bone cements and VAN–loaded CaCit addition are produced a significant decrease in the compressive strength. The result indicated that the high amount of VAN–loaded CaCit (40.8% of PMMA) was responsible for such effect, noting that the amounts of unencapsulated VAN and VAN–another carrier were 5.5 and 6.2% of the PMMA particles, respectively. More importantly, the VAN–CaCit–PMMA, which possessed an excellent drug release character, showed no change in the compressive strength after drug release. This observation indicated that the CaCit particles were probably not eluted out along with the drug. However, the particles from this work were synthesised in microscale. It implied that these particles could not be used as drug carrier for cancer treatment. Therefore, calcium citrate nanoparticles were attempted to produce as novel drug carrier in this research.

1.2.3 Encapsulated Cisplatin for chemotherapeutic drug carrier

Cancer is one of the most cause of death globally. Many treatment options for cancer exist. Chemotherapy is one of the methods which treat with chemotherapeutic agents to destroy or inhibit the growth and division of malignant cells in the treatment of cancer. The platinum-based drugs cisplatin, cis-diamminedichloroplatinum(II) (CDDP), have been widely used as chemotherapeutic agents to treat a number of cancers, e.g., testicular, ovarian, bladder, head and neck, esophageal, small and non-small cell lung, breast, cervical, stomach and prostate cancers. However, the cancer patients experience any combination of side effects. It is interesting to develop the transportation of chemotherapeutic agents to the specific cell.

Recently Thepphankulngarm[19] and co-workers had used porous silica nanoparticles (PSNs) as drug carrier. Cisplatin was encapsulated by PSNs *via* absorption to the porous. In addition, the surface of nanoparticles was added carboxylate group in order to coordinate to the cobalt center from B12 (fig. 7). The B12 grafted on the surface is an active targeting unit for tumor cells. Cisplatin releasing studies were investigated by suspending in PBS solution and stirring for 168 h (7 days) at 37°C. A white solid was observed in the solution. The product was collected by centrifugation at 10000 rpm for 30 mins. The obtained particles were refluxed in THF (60 ml) with an addition of 2–3 drops of hydrochloric acid (HCl) to remove the CTAB template. After that, the particles were washed with DI water and MeOH to obtain white particles as the product. The particles were dried under vacuum and kept in a desiccator. It should be noted that the same procedure was performed to synthesize PSNs-C of different sizes by varying the amount of DI water. Three representative nanoparticles with different particle sizes assigned as PSNs-C-250, PSNs-C-300, and PSNs-C-350 were synthesized with initial DI water volumes of 250, 300, and 350 mL, respectively. The morphologies of PSNs-C were characterized by SEM. The hydrated particle sizes of PSNs-C were analysed by DLS. The functional groups of particles were identified by FT-IR spectroscopy. The synthesis process was shown in figure 8.

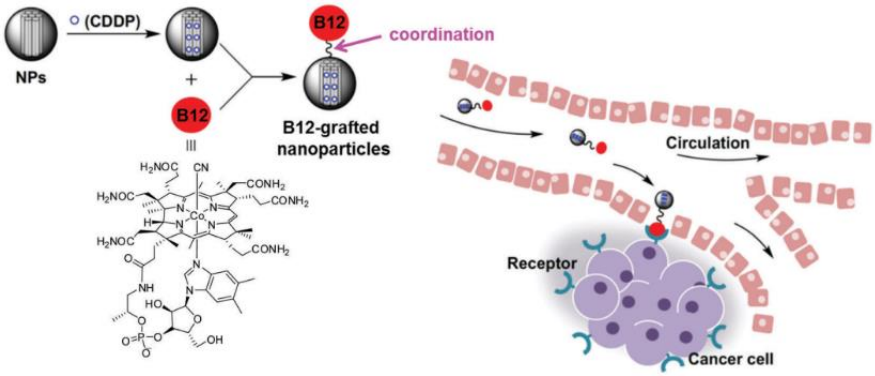


Figure 7. B12-grafted nanocarriers via coordination and proposed targeted delivery of CDDP to cancer cells.

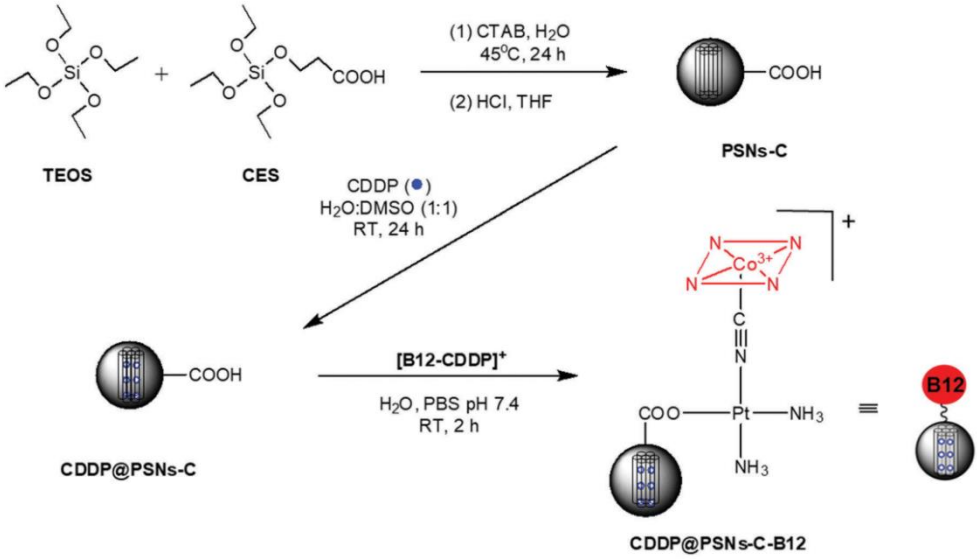


Figure 8. Synthesis of CDDP@PSNs-C-B12.

The white powder of PSNs-C became reddish, indicating that B12 was successfully grafted on the materials. The amount of $[B12\text{-}CDDP]^+$ was obtained from the calibration curve. The grafting efficiency is 20% which is calculated as the ratio between the amount of $[B12\text{-}CDDP]^+$ grafted on the particles and the amount of $[B12\text{-}CDDP]^+$ initially added. The morphologies of both PSNs-C-250 and PSNs-C-300 were not in shape since both ellipsoidal and spherical particles were observed. In

contrast, PSNs-C-350 were spherical and monodispersed as shown in figure 9a. Furthermore, the morphology of CDDP@PSNs-C-B12 showed spherical in shape and monodispersed nanoparticles (fig. 9b)

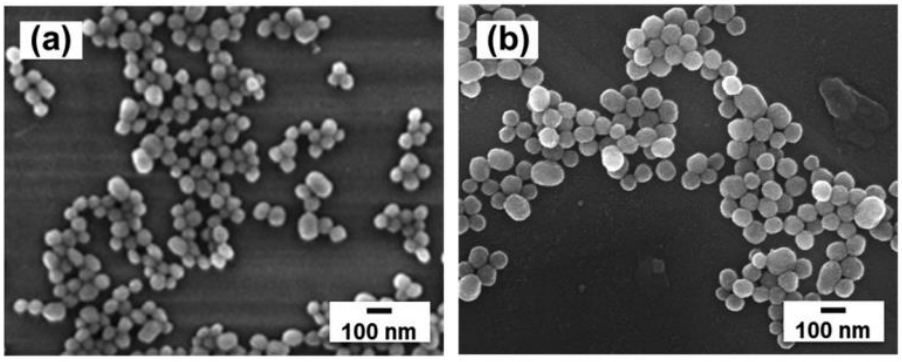


Figure 9. SEM images of a) PSNs-C and b) CDDP@PSNs-C-B12.

However, porous silica nanoparticles in this work are unable to dissolve in any solvents. This is the great barrier for the nanoparticles to be applied in cancer treatments.

1.3 Objectives

To synthesise and characterise CaCit nanoparticles which encapsulated cisplatin and grafted on the surface of the nanoparticles by vitamin B12.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Anhydrous calcium nitrate (≥ 98.0%) and trisodiumcitrate dihydrate (≥ 99.0%) were obtained from Merck (Germany). Cisplatin was purchased from Sigma–Aldrich (St. Louis, MO). Phosphate Buffered Saline (PBS buffer) was purchased from VWR Chemicals (Vienna, Austria). Vitamin B12 (≥95%) was purchased from Merck.

2.2 Instruments

Scanning electron microscope (SEM) JEOL series JSM-6480LV

ImageJ software for SEM image processing and analysis.

Thermogravimetric analyzer (TGA) STA 409 PC TA system.

Fourier transform infrared spectroscopy (ATR FT-IR) Thermo scientific Nicolet 6700

LSM 800 Confocal microscope

HORIBA labRAM HR800 Raman spectrometer

Thermo Scientific-Model iCAP 6500 series ICP-OES spectroscopy

2.3 Methods

2.3.1 Preparation of calcium citrate-based cisplatin nanoparticles (CaCit@CDDP)

2.3.1.1 Preparation of calcium citrate-based cisplatin nanoparticles (CaCit@CDDP) using Ca^{2+} source from $CaCl_2$

CaCit@CDDP nanoparticles were prepared by co-precipitation between calcium ions and citrate ions with varied concentrations of CDDP as shown in Table 1. In a 15-mL centrifuge tube, 2 mL of 1.5 M $CaCl_2$ was mixed with various amount of 2.0% (w/v) CDDP (in H_2O : DMSO = 1:1). During the vigorously vortex, 375 µl CDDP solution (condition 1) was added in to $CaCl_2$ solution (15 mL centrifuge tube). After that, 2 mL of 1.5 M trisodium citrate dihydrate was added into the mixture. The reaction mixture was kept vigorously vortexed for 10 minutes and left rocking for 8 and 18 hours as shown in the table 1. Finally, 5 mL of DI water was added during vortex to disperse the particle. The suspension was centrifuged (65000 rpm, 10 mins) to remove the supernatant until it was cleared. The white powder was collected by freeze-drying.

Table 1. The percentages of encapsulation of CDDP and rocking time.

Condition	CDDP2.0% (w/v) (µl)	CDDP (%)	Rocking time (hours)
1	375	1.25	18
2	600	2.50	18
3	375	1.25	8
4	600	2.50	8
5	1000	3.33	8
6	2000	6.66	8

2.3.1.2 Preparation of calcium citrate-based cisplatin nanoparticles (CaCit@CDDP) using Ca^{2+} source from $Ca(NO_3)_2$

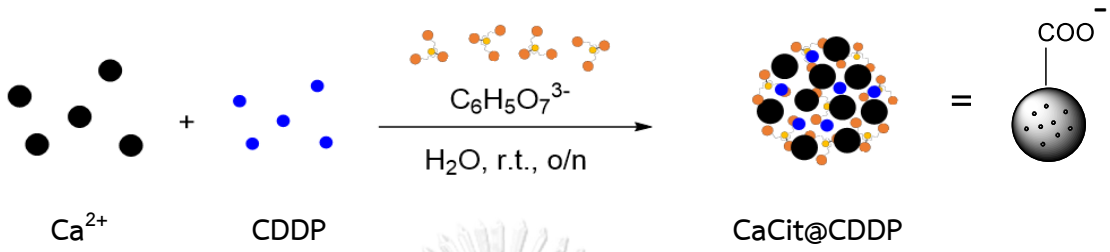


Figure 10. Synthesis of CaCit@CDDP.

CaCit@CDDP nanoparticles were prepared by co-precipitation (fig. 10) between calcium ions and citrate ions with varied concentrations of CDDP as shown in Table 2. In a 15-mL centrifuge tube, 2 mL of 1.5 M $Ca(NO_3)_2$ was mixed with various amount of 4.0% (w/v) CDDP (in H_2O : DMSO = 1:1). During the vigorously vortex, 1426 µl CDDP solution (condition 7) was added in to $Ca(NO_3)_2$ solution (15 mL centrifuge tube). After that, 2 mL of 1.5 M trisodium citrate dihydrate was added into the mixture. The reaction mixture was kept vigorously vortexed for 10 minutes and left rocking for 4 hours. Finally, 5 mL of DI water was added during vortex to disperse the particle. The suspension was centrifuged (65000 rpm, 10 mins) to remove the supernatant until it was cleared. The white powder was collected by freeze-drying.

Table 2. The percentages of encapsulation of CDDP and rocking time.

Condition	CDDP 4.0% (w/v) (µl)	CDDP (%)	Rocking time (hours)
7	1426	10.0	4
8	2853	20.0	4

2.3.2 Preparation of calcium citrate-based vitamin B12/cisplatin nanoparticles (CaCit@CDDP-B12)

2.3.2.1 Synthesis of B12-CDDP conjugates ($[B12\text{-}CDDP]^+$)

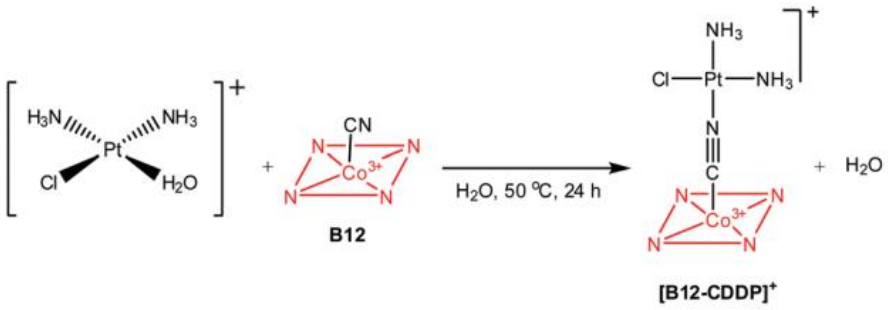


Figure 11. Synthesis of $[B12\text{-}CDDP]^+$ as active targeting unit.

In a 50-ml beaker, 0.5 mmol of both CDDP and $AgNO_3$ were dissolved in 13.6 mL of DI water. The mixture was stirred at room temperature for 2 hours. The Silver chloride by product was removed by centrifugation. Then 0.5 mmol of B12 was added into the remaining solution and kept stirring at 50°C for 24 h. After that, the solvent was removed under vacuum to obtain a red powder as a product with 95% yield. The synthesis diagram of $[B12\text{-}CDDP]^+$ was shown in figure 11.

2.3.2.2 Synthesis of CaCit@CDDP-B12[19]

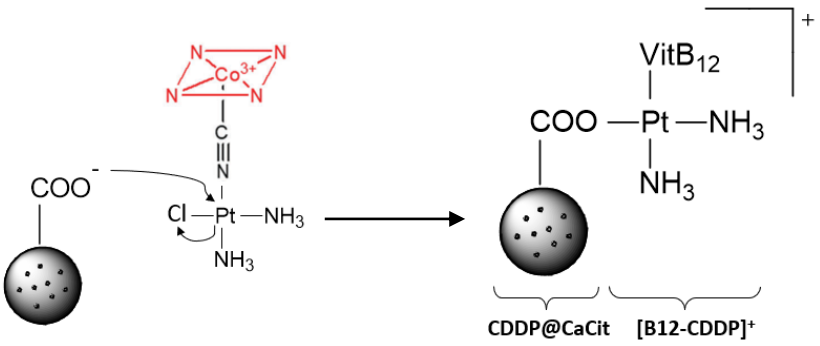


Figure 12. Synthesis of CaCit@CDDP-B12.

In a 100-ml beaker, 0.5 mmol of $[B12\text{-}CDDP]^+$ was dissolved in 50 mL of PBS solution (pH 7.4) and stirred at room temperature for 15 min. Then 0.5 mmol of CaCit@CDDP was added to the solution. The reaction was continuously stirred for 2 hours. The white powder of CaCit@CDDP became reddish, indicating that B12 was successfully grafted on the materials. The particles were collected by centrifugation at 65000 rpm for 10 mins. After that, the particles were washed with DI water and freeze dried under vacuum overnight. The synthesis diagram of CaCit@CDDP-B12 was shown in figure 12.

The white powder, CaCit@CDDP, was collected with approximately 85% yield. After the surface of the nanoparticles were modified by vitamin B12, the reddish powder was observed as CaCit@CDDP-B12 with 67% yield. The obtained size of the spherical nanoparticles is approximately 553.0±65.0 nm.

2.3.3 Cisplatin release study

2.3.3.1 Cell culture

A549 cell was purchased from the American Type Culture Collection, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All cells were incubated in a humidified incubator at 37°C in 5% CO_2.

2.3.3.2 Cell viability assay

Cell viability was analyzed using the PrestoBlue reagent. A549 cells were cultured in 96-well plates and to 80% confluence. A549 cells were treated with CDDP for 48 h. After incubation, PrestoBlue reagent (10 ul) was added to each well according to the manufacturer's protocol, followed by a further 30 min incubation under the same incubator conditions. Following this, the plates were then analyzed on a microplate reader to determine the absorbance of the samples. The mean optical density in the indicated groups was used to calculate the percentage of cell viability.

CHAPTER III

RESULTS AND DISCUSSION

The synthesis of calcium citrate-based cisplatin nanoparticles (CaCit@CDDP) was prepared *via* simple chemical co-precipitation methods from calcium ion, citrate ion and cisplatin (CDDP). Carboxylic group on the surface of calcium citrate nanoparticle has been linked to platinum core in order to coordinate with vitamin B12 (B12). The morphology of nanoparticles was characterised by SEM. The results of encapsulation and surface modification were analyzed by TGA, ICP-OES and Raman. Moreover, CDDP releasing study was investigated *via* Prestoblue viability assay. CaCit@CDDP-B12 decreased the cell viability of A549 cell line much significant than the cells treated with CDDP, whereas CaCit alone had no effect on cell viability.

3.1 SEM and temperature profile of Calcium citrate-based cisplatin nanoparticles (CaCit@CDDP) and calcium citrate-based vitamin B12/cisplatin nanoparticles (CaCit@CDDP-B12)

3.1.1 Using $CaCl_2$ as Ca^{2+} source for synthesis of CaCit@CDDP

In the process of encapsulation, co-chemical precipitation method was used to synthesize CaCit@CDDP nanoparticles. This reaction used $CaCl_2$ as Ca^{2+} source to produce CaCit. Furthermore, the 6 varied conditions of rocking time and concentration of CDDP had been done to obtain a nano-scale range of CaCit@CDDP.

Based on previous study, the synthesis of CaCit nanoparticles was used 18 hours as rocking time. Therefore, the synthesis of CaCit@CDDP were commenced by using the same rocking time as shown in the table 3.

3.1.1.1 Condition 1 and 2

Table 3. Molar ratio of calcium citrate encapsulated CDDP: Condition 1 and 2.

Condition	$CaCl_2$ (mM)	$Na_3C_6H_5O_7$ (M)	CDDP (%)	Rocking time (hours)	Size (nm)
1	1.5	1.5	1.25	18	5380
2	1.5	1.5	2.50	18	4850

In condition 1 (Table 3), the reaction was rocked for 18 hours. The particle morphology was determined by SEM. SEM image showed that CaCit@CDDP of condition 1 were not uniform in nano-scale as shown in figure 13. The product obtained as aggregated thick nanosheets. By increasing 1.25% to 2.50% loading of CDDP in condition 2, both nanosheets and nanoneedles were formed (fig. 14). This may because crystallisation time was too long for the reaction which led CaCit to be

the most thermodynamically stable form[9, 20]. It indicated that crystallization process depends on rocking time of the reaction. As this result, rocking time was changed for the next reaction as shown in the table 4.

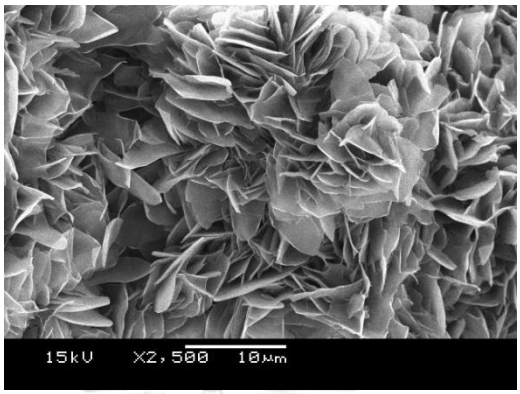


Figure 13. SEM of Calcium citrate Encapsulated CDDP: Condition 1.

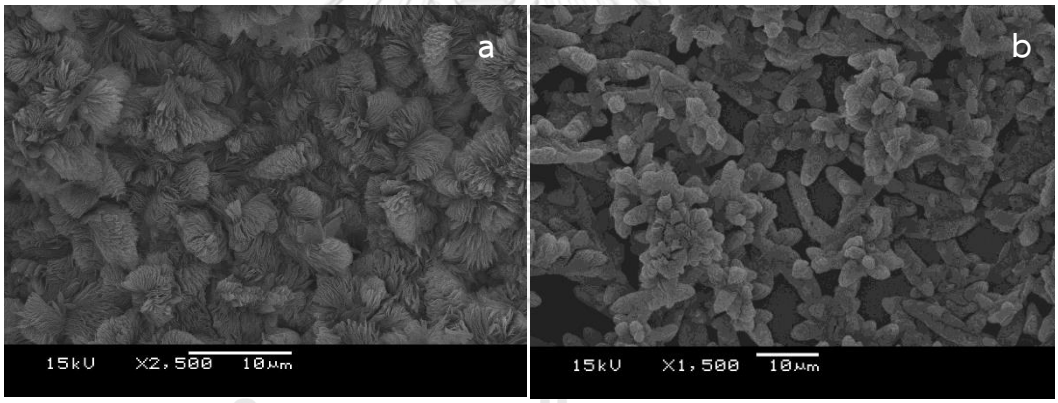


Figure 14. SEM of Calcium citrate Encapsulated CDDP: Condition 2.: a) sheet form and b) needle-like crystals.

3.1.1.2 Condition 3 and 4

Table 4. Molar ratio of Calcium citrate Encapsulated CDDP: Condition 3 and 4.

Condition	$CaCl_2$ (mM)	$Na_3C_6H_5O_7$ (M)	CDDP (%)	Rocking time (hours)	Size (nm)
3	1.5	1.5	1.25	8	774.7
4	1.5	1.5	2.50	8	757.1

For condition 3 and 4, percentage of encapsulated CDDP was similarly to condition 1 and 2 respectively but rocking time was changed to 8 hours. The morphology had changed. SEM image of the particles synthesized from condition 3 and 4 showed smaller needle-like calcium citrate. The result shows a decrease in particle size tremendously from 4850 nm to 757.1 nm as shown in figure 15. It indicated that the obtained size was in nano-scale with at least 8 hours of rocking time. In order to obtain the optimized condition, percentage of loading CDDP was increased to 3.33% and 6.66% (table 5).

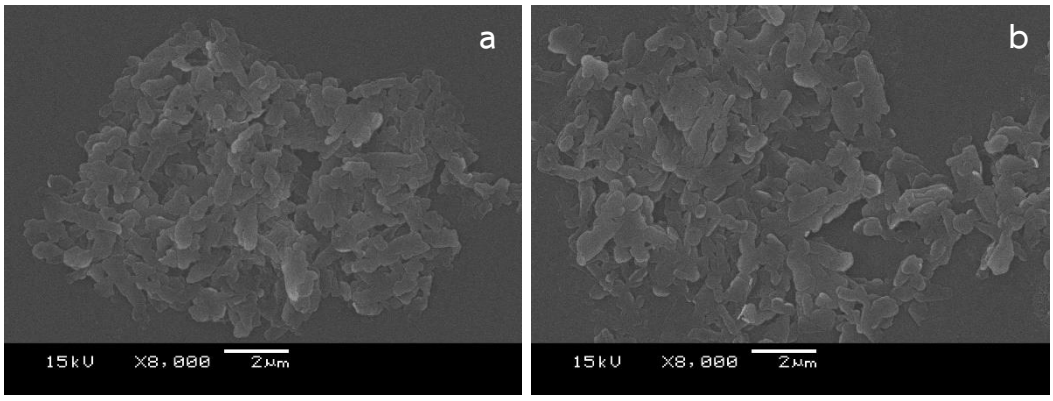


Figure 15. SEM of Calcium citrate Encapsulated CDDP: Condition 3 (a) and 4 (b).

3.1.1.3 Condition 5 and 6

Table 5. Molar ratio of Calcium citrate Encapsulated CDDP: Condition 5 and 6.

Condition	$CaCl_2$ (mM)	$Na_3C_6H_5O_7$ (M)	CDDP (%)	Rocking time (hours)	Size (nm)
5	1.5	1.5	3.33	8	780.6
6	1.5	1.5	6.66	8	581.5

In this experiment, percentage of encapsulated CDDP was slightly increased to 3.33 % and 6.66 % assigned to condition 5 and 6 respectively. The small needle-like and spherical calcium citrate nanoparticles were observed in condition 5 (fig. 16a and 16b). The obtained size of spherical nanoparticles is 780.6 nm. In addition, the spherical nanoparticles were also obtained in condition 6 (fig. 16c and 16d) which the size is around 581.5 nm. This result confirmed that a slight increase of CDDP content directly affects the morphology and crystallization process of calcium citrate nanoparticles.

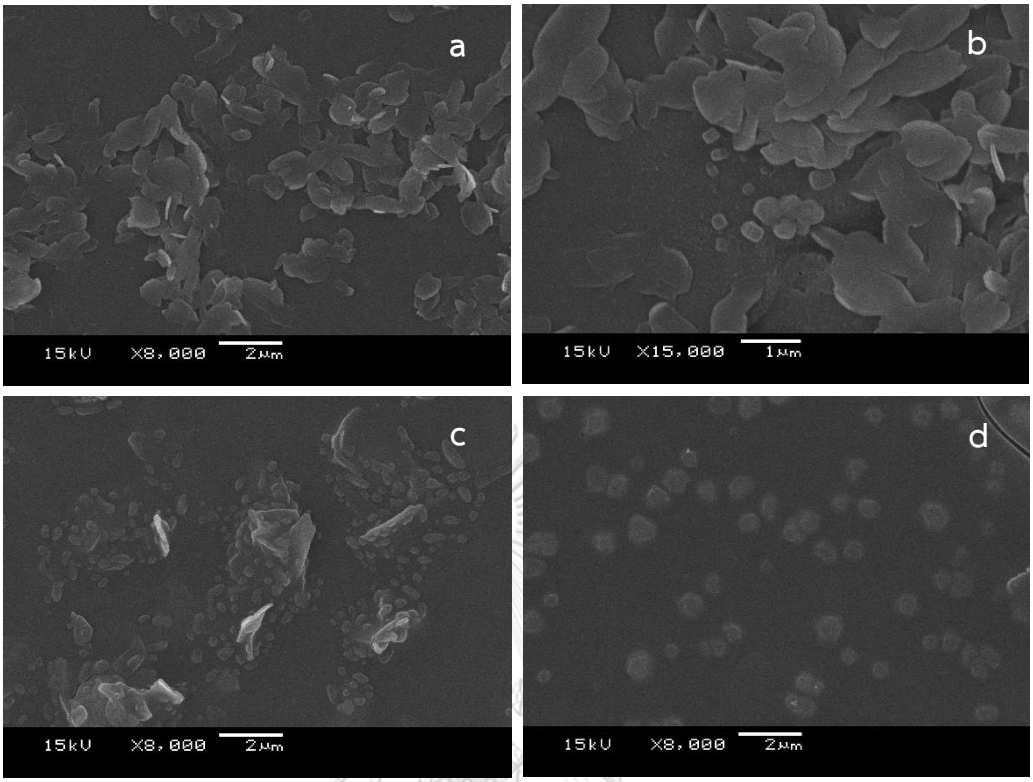


Figure 16. SEM of Calcium citrate Encapsulated CDDP: Condition 5 (a and b) and 6 (c and d).

After the morphology and the obtained size of the nanoparticles are suitable, the product was conducted by TGA to investigated percentage of loaded CDDP as shown in figure 17.

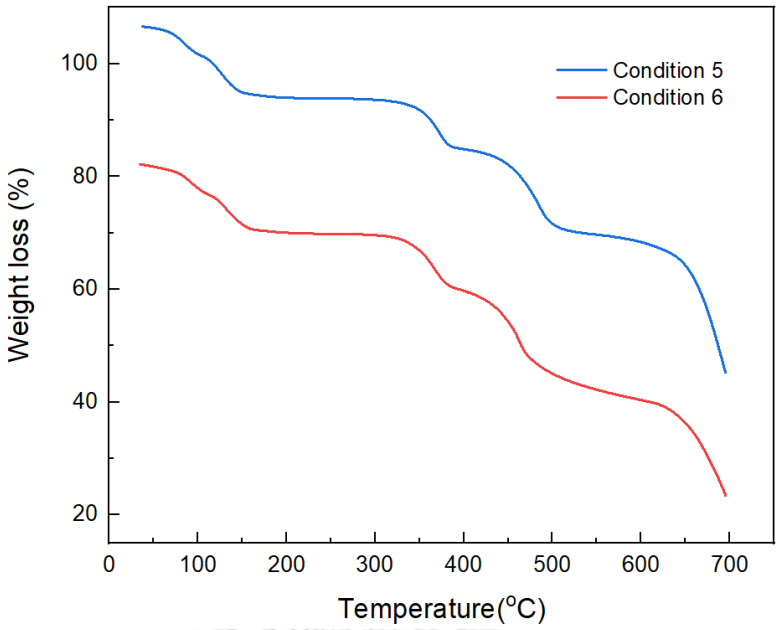


Figure 17. Temperature profile of CaCit@CDDP: condition 5 and 6.

The temperature profile of the products from condition 5 and 6 is definitely the same as that of pure calcium citrate (CaCit) (fig. 17) Firstly, 2 steps mass loss at 80-120°C corresponded to the surface-adsorbed water molecules and water in the crystal molecules of CaCit[9]. The second weight loss also has 2 steps around 350-480°C which assigned to the decomposition of CaCit. into $CaCO_3$. Then, $CaCO_3$ was decomposed at 650°C and changed to CaO in the last step. This result indicated that CDDP was not encapsulated by CaCit nanoparticles because decomposition peak of CDDP did not appear in the temperature profile that should be around 250-300°C[21].

In the previous experiment, we noticed that the transparency of $CaCl_2$ solution is changed to yellow color and also found some sediment after adding CDDP solution. The increase in loading percentage of CDDP might affect the solubility in CaCl2 media, hence the unwanted recombination of CDDP itself occurred. It could be the reason that CDDP signal does not exist in the temperature profile (fig. 17). Thus, the synthesis of CaCit was changed Ca^{2+} source to $Ca(NO_3)_2$ in order to study the effect of negative charge in the reaction.

3.1.2 Using $Ca(NO_3)_2$ as Ca^{2+} source for synthesis of CaCit@CDDP

In this method, $Ca(NO_3)_2$ was used as calcium ion source and concentration of CDDP also considerably increased from 6.6% to 10% (condition 7) and 20% (condition 8). The morphology of the sample in both conditions showed evolution of calcium citrate nanoparticles as shown in figure 18.

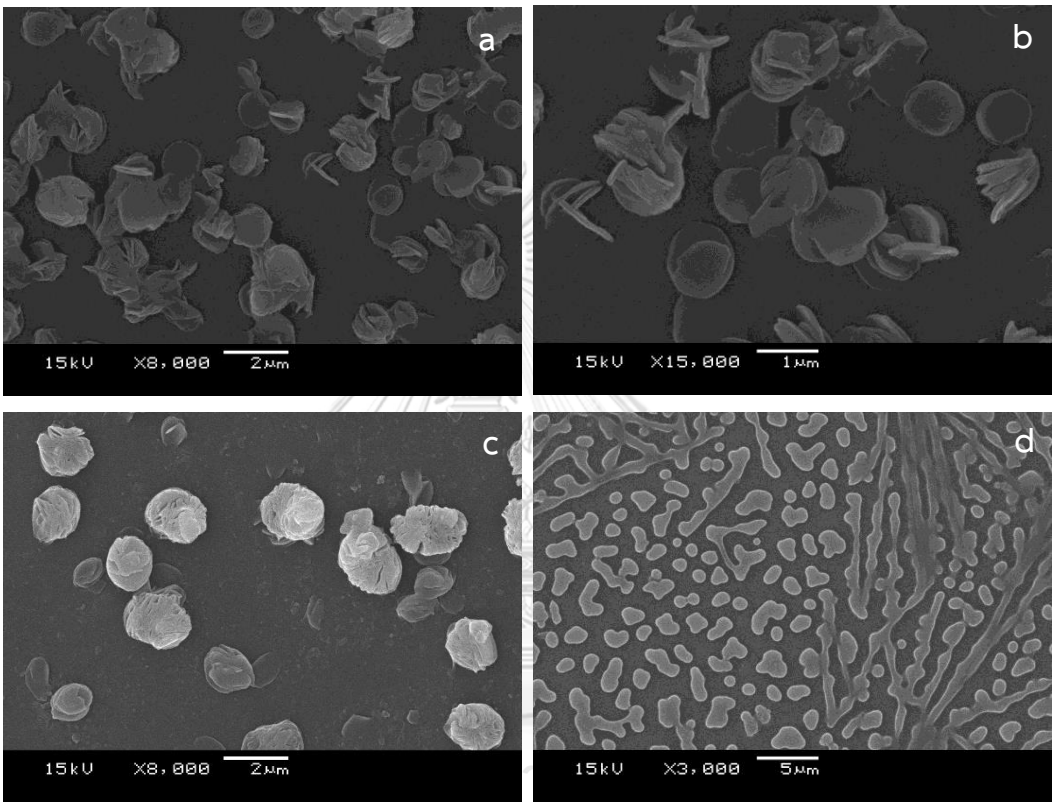
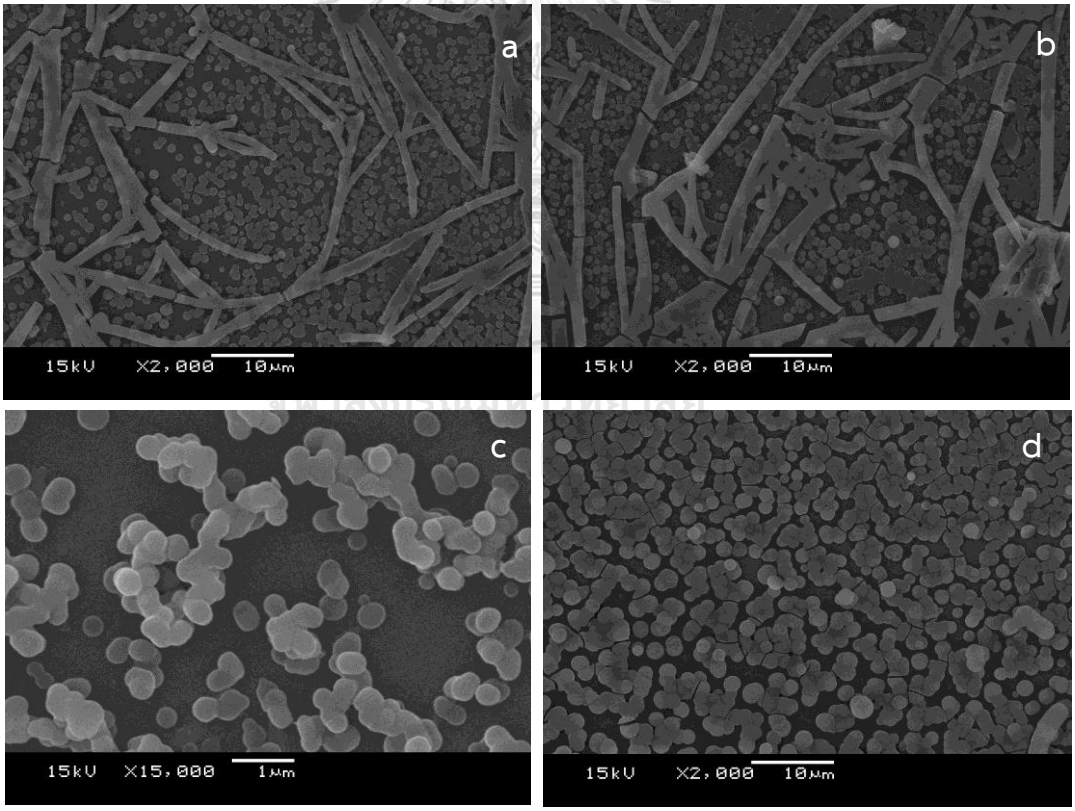


Figure 18. SEM of Calcium citrate Encapsulated CDDP: Condition 7 (a and b) and 8 (c and d).

Interestingly, the morphology of calcium citrate entirely became spherical nanoparticles when using $Ca(NO_3)_2$ as Ca^{2+} source. This result showed negative ions (NO_3^-) from Ca^{2+} source affect the formation of CaCit@CDDP nanoparticles. This may because CDDP exchange from Cl^- to H_2O leading to the formation of monoaqua cationic complex $[PtCl(NH_3)_2(H_2O)]^+$ or the diaqua complex $(Pt(NH_3)_2(H_2O)_2)$[19]. In the first case, $CaCl_2$ was used as Ca^{2+} source and CDDP was dissolved in DI water/dimethylsulfoxide (DMSO) (1:1 v/v) which mean CDDP transformed to cis-diamminediaqua platinum(II) because of ligand substitution by water. When the

reaction mixture was rocked, chloride ions from $CaCl_2$ recombined (substituted with H_2O ligand) with platinum then cis-diamminedichloroplatinum (II) was produced and the characteristic yellow color of starting CDDP was observed. This incidence could disturb the formation of the nanoparticles. This could be a reason that the morphology showed sheet form, needle-like crystal and also microparticles in previous condition. By contrast, the spherical nanoparticles were observed or produced in condition 7 and 8. These particles were collected after the reaction was rocked for just 4 hours. Due to the spherical particles from condition 7 and 8 were observed and condition 8 had used more % loaded CDDP than condition 7, lead to the reaction in condition 8 was collected the product at every hour because the particles tend to be spherical based on previous study before rocking time has reached to 4 hours. The morphology of these products was shown in figure 19.



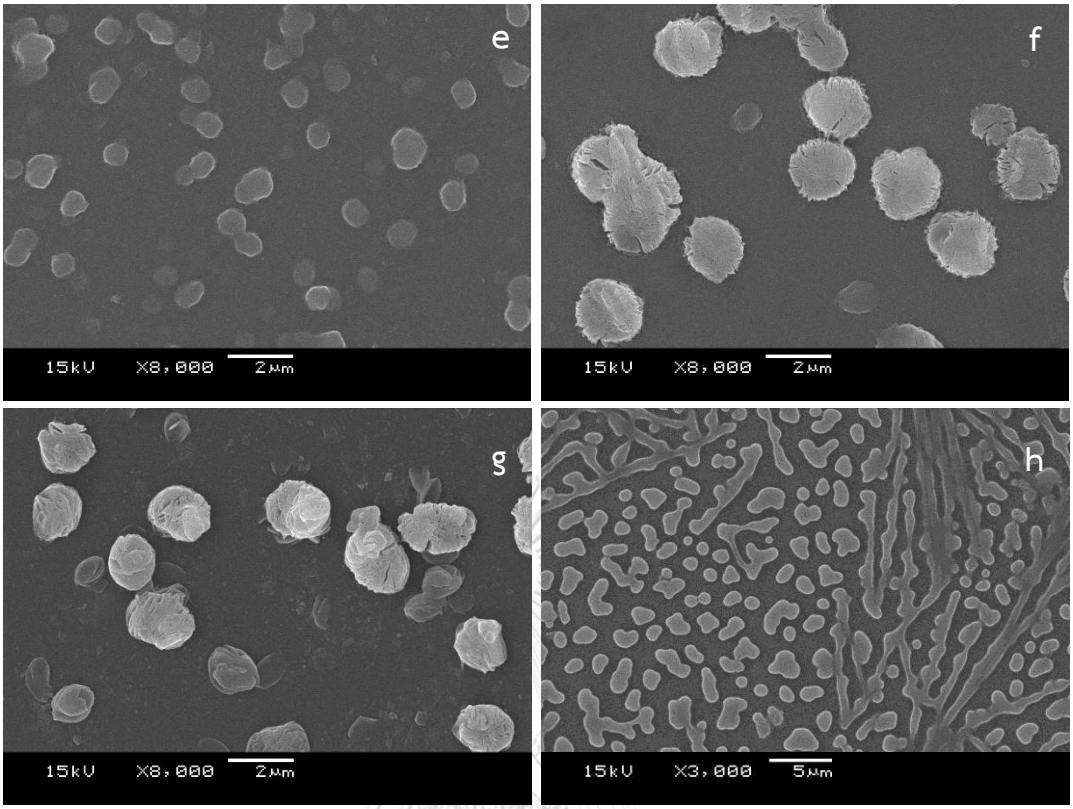


Figure 19. SEM of Calcium citrate Encapsulated CDDP from condition 8 by time dependence: 1 hr. (a and b), 2 hrs. (c and d), 3 hrs. (e and f) and 4 hrs. (g and h).

From the earlier study[22], the morphology of CaCit were rod at the beginning then the particles were changed to nano-scale. Finally, the aggregated thick nanosheets and nanoneedles were produced as the most thermodynamically stable form. This experiment is in accordance with previous study as shown in figure 19. The morphology of CaCit@CDDP at different rocking time in the reaction showed that after the reaction was rocked for an hour, the particles form mixture between spherical CaCit nanoparticles and needle-like CaCit as shown in figure 19a and 19b. Afterward, the particles are completely transformed to spherical nanoparticles at 2 hours rocking time. The obtained size (fig. 19c-d) of spherical nanoparticles is 369.9 ± 42.8 nm. Then, the particles became thicker after rocked for 3 hours. The size (fig. 19e-f) is around 1234 nm. Finally, the product became micro particles and needle-like CaCit crystals at 4 hours rocking time (fig. 19g-h). From this observation, It can be confirmed that rocking time affect directly to the morphology of CaCit. The longer

rocking time can make CaCit to became the most thermodynamically stable form (needle-like crystals). Additionally, the temperature profile of condition 8 synthesised by using $Ca(NO_3)_2$ as Ca^{2+} source clearly showed decomposition peak of CDDP (fig. 20). The additional weight loss at 329.55°C corresponding to CDDP appeared in temperature profile of CaCit@CDDP which is approximately 7.1%.

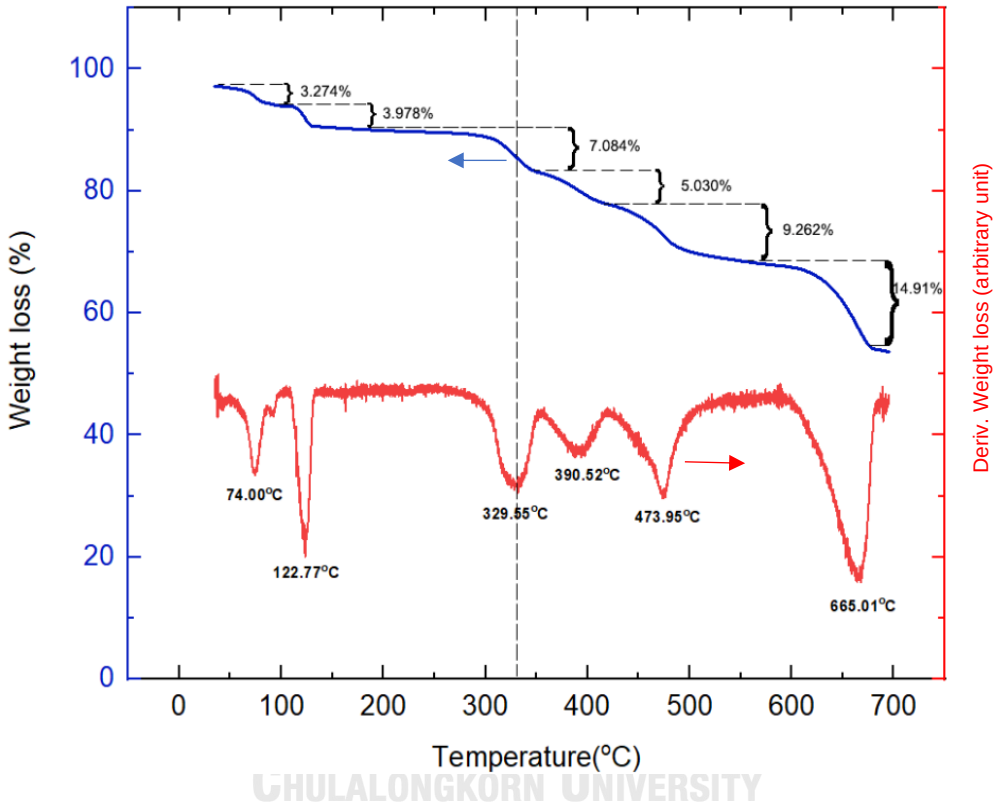


Figure 20. Temperature profile of CaCit@CDDP: condition 8.

3.1.3 Calcium citrate-based vitamin B12/cisplatin nanoparticles (CaCit@CDDP-B12)

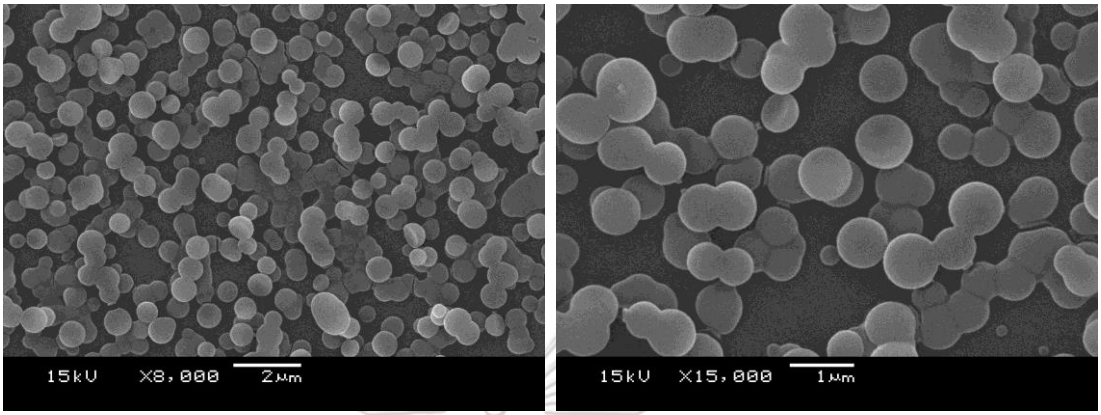


Figure 21. SEM of Calcium citrate-based vitamin B12/CDDP nanoparticles (CaCit@CDDP-B12).

After characterisation, the product from condition 8 was selected to perform surface modification with B12. The final products were synthesised *via* coordination reaction. B12 was decorated on the surface of CaCit@CDDP nanoparticles by ligand substitution. SEM images (fig. 21) showed spherical nanoparticles. The obtain size of CaCit@CDDP-B12 is 553.0±65.0 nm. This result indicated that the appearance of final nanoparticles was insignificantly changed in the aspect of both morphology and size after surface modification with B12. Moreover, TGA result of CaCit@CDDP-B12 was shown in figure 22.

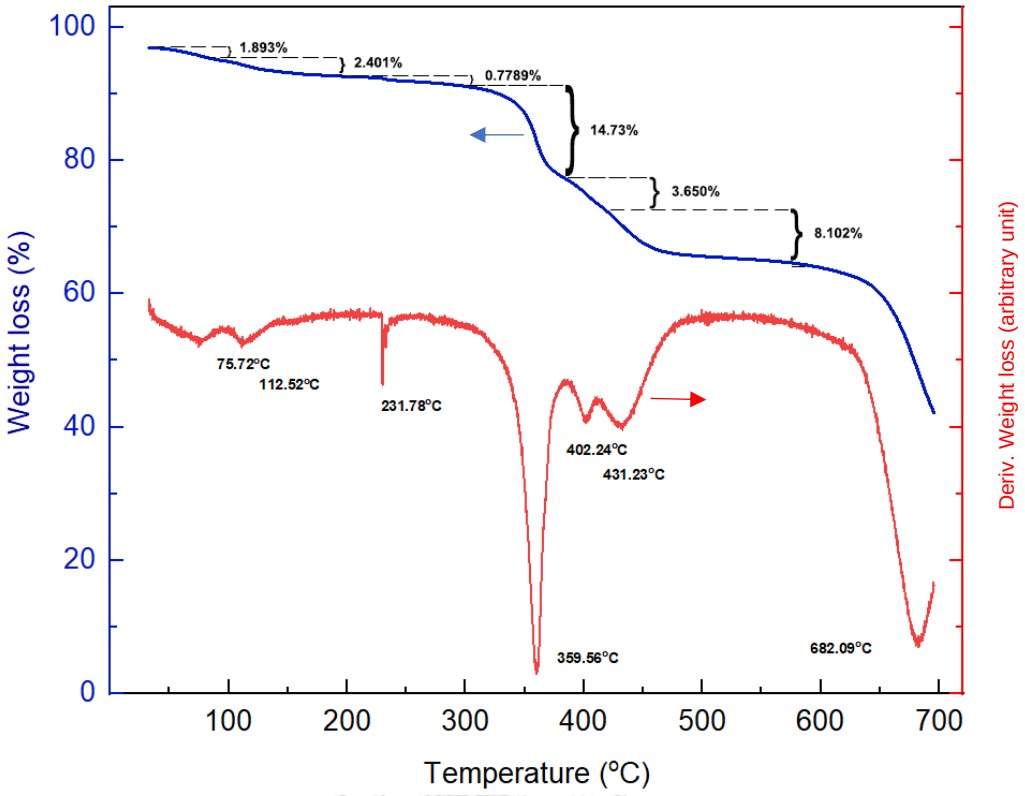


Figure 22. Temperature profile of CaCit@CDDP-B12.

Temperature profile of CaCit@CDDP-B12 (fig. 3.22) showed that B12 was evidently incorporated with CaCit@CDDP nanoparticles. The weight loss showed only 0.78% appeared at 231.78°C corresponding to B12 grafted on the nanoparticles. In fact, cyano group from cyanocobalamin can be converted into cyanide at 140-145°C but this result showed significantly decomposition temperature shifted. This may causes by a strong interaction between cyano group and CDDP. Additionally, the decomposition signal of CDDP also shifted from 329.55°C to 359.55°C because of B12-CDDP conjugated ([B12-CDDP]$^{+}$) which is on the surface of nanoparticles. It also implied that chemical interaction had occurred on the nanoparticles. Thus, percentage of CDDP loadings are significantly increased from 7.1% to 14.7%.

3.2 The study of the interior of the nanoparticles

The optical analysis was investigated for studying internal structure of CaCit nanoparticles. In this work, UV-visible can be used to determine excitation wavelength that should properly apply in Raman.

3.2.1 UV-Visible spectroscopy

Absorbance spectra of all products, substrates and solvent were recorded. The results are shown in figure 23.

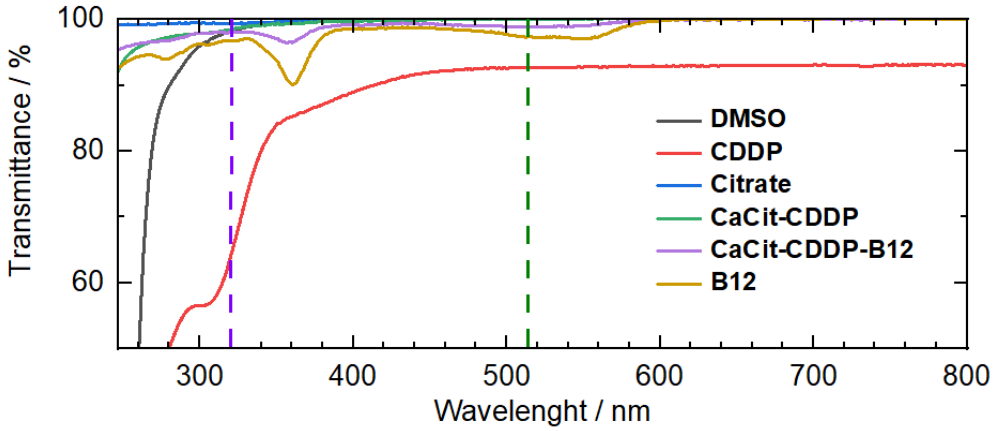


Figure 23. Absorbance spectra of all chemical products.

B12 showed weak absorption around 500 to 580 nm and stronger absorption around 320 to 380 nm. Additionally, CDDP also showed broad absorption from 300 to 450 nm. As a result, green laser (514 nm) and UV laser (325 nm) were used to excite B12 in the product. Moreover, citrate did not absorb any wavelength of light source but CaCit-CDDP showed broad absorption (green line) from 300 to 380 which is corresponding to CDDP. In addition, CaCit-CDDP-B12 characteristically showed absorption of B12 moiety around 500 to 580.

3.2.2 Raman spectroscopy

According to UV-Vis spectroscopy results, Raman spectra of CDDP were excited with the 325 and 514 nm. laser as shown in figure 24.

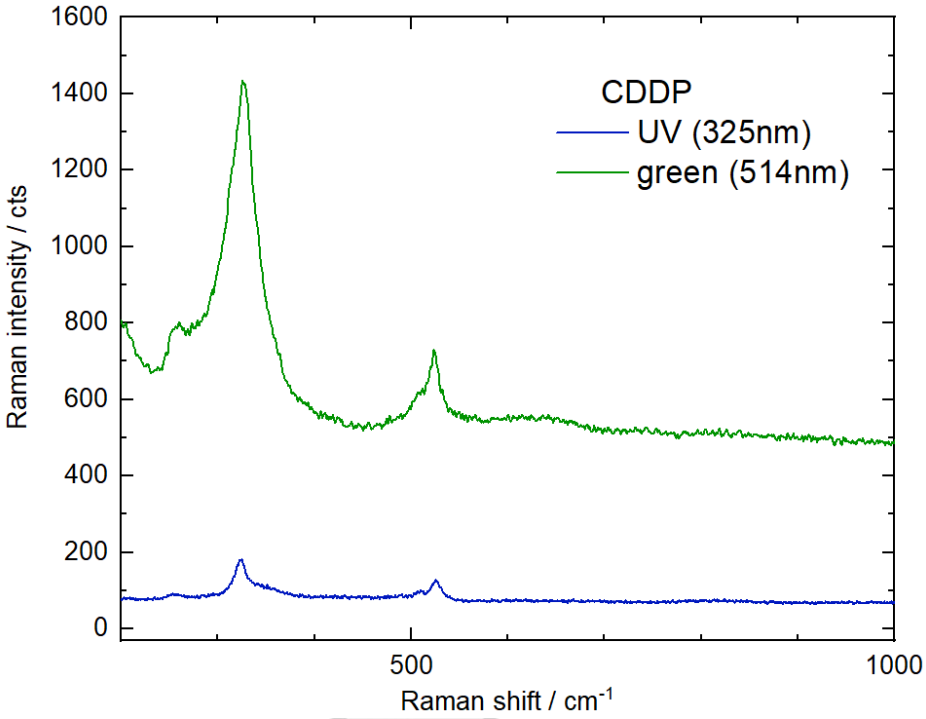


Figure 24. Raman spectra of CDDP.

Using green laser excitation, the *ν*(Pt–Cl) asymmetric stretching vibrations were observed around 322 cm^{-1} as an intense band[23]. On the other hand, the *ν*(Pt–Cl) symmetric stretching vibrations were observed around 522 cm^{-1} with weaker intensity[23]. Using UV laser excitation, Raman spectra showed the same characteristic signals but in much weaker intensities. It is because the parameters e.g. detector and light power from both lasers are different.

According to UV-Vis spectroscopy result, Raman spectra of B12 were excited with the 325 and 514 nm. laser as shown in figure 25.

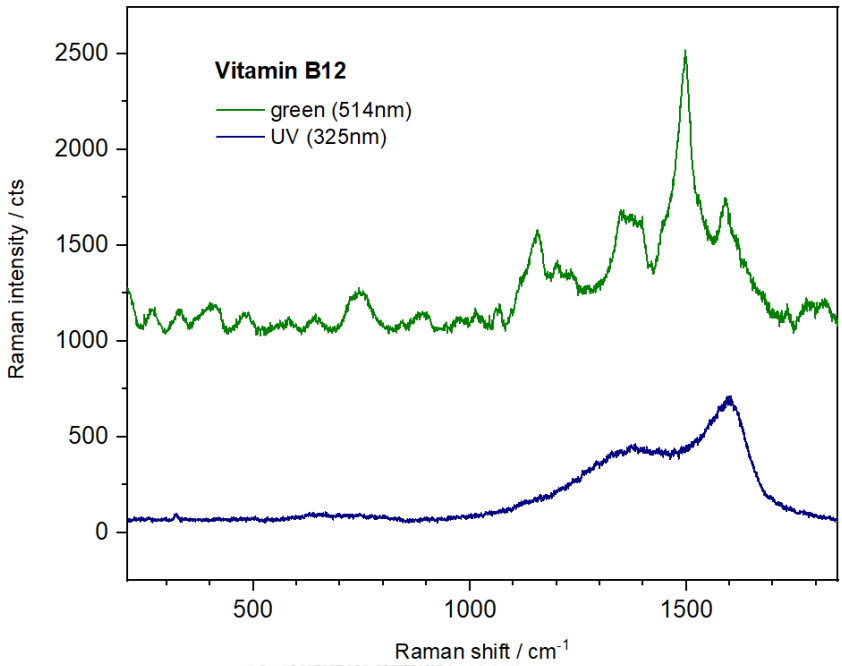


Figure 25. Raman spectra of vitamin B12.

Using green laser excitation, the ring stretching modes of heterocycles were observed around 1500 cm^{-1} as an intense band[24]. Using UV laser excitation, the ring stretching modes of heterocycles were observed blue shifted to around 1583 cm^{-1} with broader signal. This is due to the partial decomposition of organic compound under prolonged UV treatment[25]. Moreover, it is because of stronger absorption of B12 around 320 to 380 nm in UV-Vis spectroscopy.

To prove the hypothesis that CDDP could be encapsulated inside CaCit nanoparticles and B12 were grafted on the surface of CaCit nanoparticles, the experiment was designed to synthesise a series of CaCit@CDDP nanoparticles with various thickness of citrate outer layers. Five different thicknesses of citrate outer layer were grown up from the starting CaCit@CDDP nanoparticles using the same method developed earlier[26].

In brief, 100 mg of CaCit@CDDP nanoparticles generated by using condition 8 with 2 hours rocking time were dispersed in 2 mL of DI water. Then, 2 mL of 1.5 M

trisodium citrate dihydrate was added. The mixture was stirred for 4 hours. Samples were collected at 1, 2, 3 and 4 hours. After washed and dried the CaCit@CDDP2, CaCit@CDDP3, CaCit@CDDP4 and CaCit@CDDP5 with different thickness of citrate outer layer were obtained as white powder.

SEM images of the series were shown in figure 26. The average sizes of CaCit@CDDP2, CaCit@CDDP3, CaCit@CDDP4 and CaCit@CDDP5 were measured to be 859.8, 1304.4, 2304.3 and 3649.5 nm, respectively comparing with the original CaCit@CDDP of 369.9 nm. As expected, the longer reaction time, the bigger nanoparticles were produced.

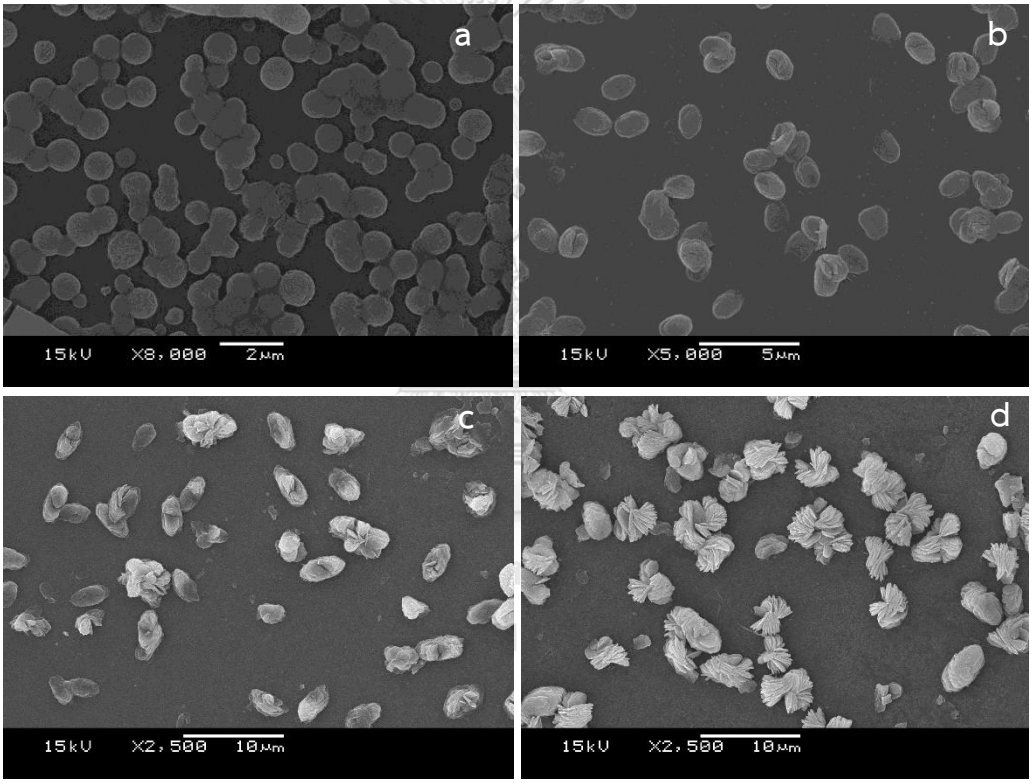


Figure 26. SEM of the nanoparticles series : CaCit@CDDP2(a), CaCit@CDDP3(b), CaCit@CDDP4(c) and CaCit@CDDP5(d).

Raman spectra of the CaCit@CDDP series were investigated by applying UV laser with 450 μW. Figure 27 showed that only C=O stretching at 1613 cm^{-1} was observed for the whole series despite the thickness of the citrate outer layer. Moreover, this intensity of C=O stretching signal from citrate ion was increased along

with the thickness of the outer layer. Surprisingly, the characteristic Raman signal of CDDP around 300-500 cm^{-1} did not exist (fig. 27). Therefore, the laser power was increased. The Raman spectra were re-recorded as shown in figure 28.

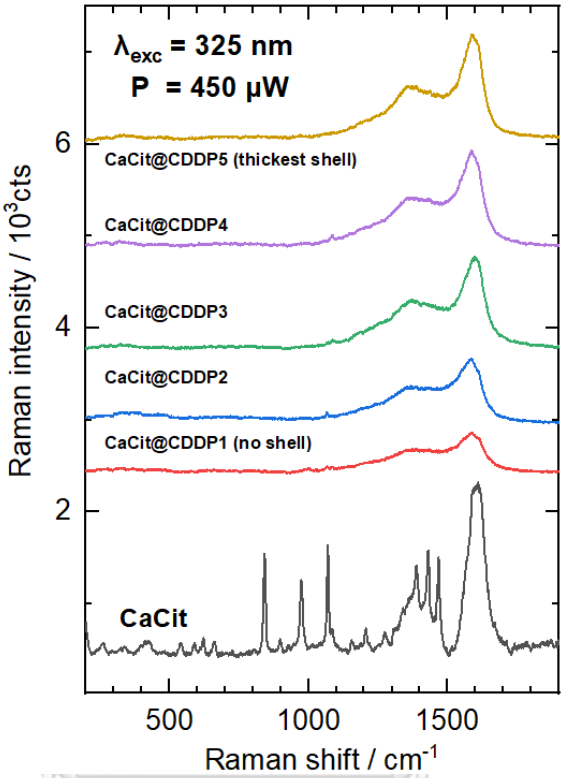


Figure 27. Raman spectra of the nanoparticle series with 450 μW of UV (325 nm) laser.

As expected, asymmetric *ν*(Pt–Cl) stretching vibrations and symmetric *ν*(Pt–N) stretching vibrations respectively at 322 cm^{-1} and 522 cm^{-1} were detected with the UV laser power 4.5 mW (10 times stronger, fig. 28). The Raman spectrum of CaCit@CDDP1 (original CaCit@CDDP without citrate outer layer) exhibited both CDDP and citrate vibration signals. The fact that CDDP was still observed in other cases with different thickness of citrate outer layer demonstrates the existence of CDDP on the surface of the nanocomposite and hence denies the encapsulation hypothesis. Moreover, the signal of *ν*(Pt–Cl) stretching vibrations are blue shifted. It implied that the bigger nanoparticles, the less CDDP encapsulations were occurred.

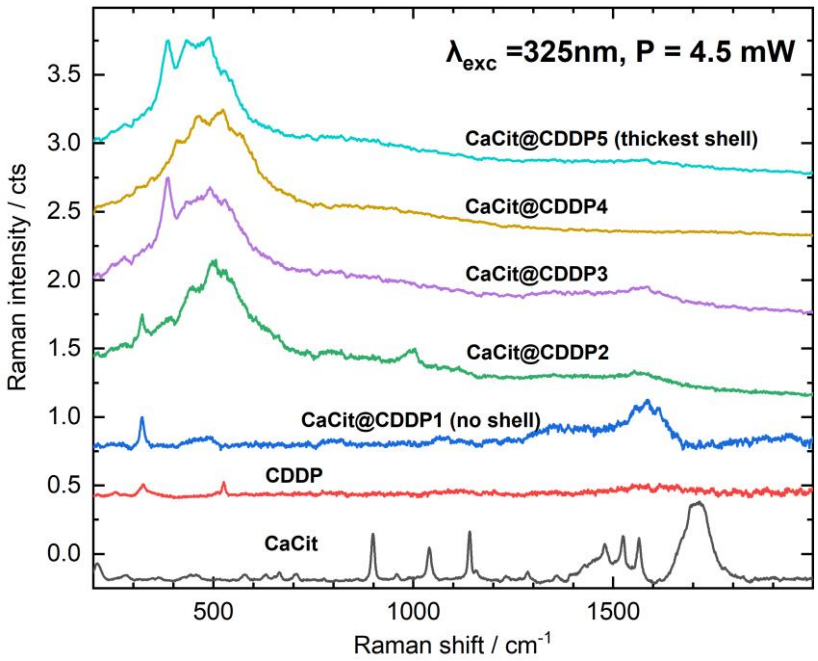


Figure 28. Raman spectra of the nanoparticle series with 4.5 mW of UV (325 nm) laser.

The spectra from figure 27 and 28 were included in this spectrum (fig. 29). The spectrum (blue line) was started measuring by 450 μW UV (325 nm) laser. Then, a sample was treated with 4.5 mW as a power (green line). Finally, 450 μW of the laser were used to measure a sample as shown in black line. The signals corresponding to CDDP were obviously revealed in this spectrum. This is because a sample was heated and damaged by UV (325 nm) laser.

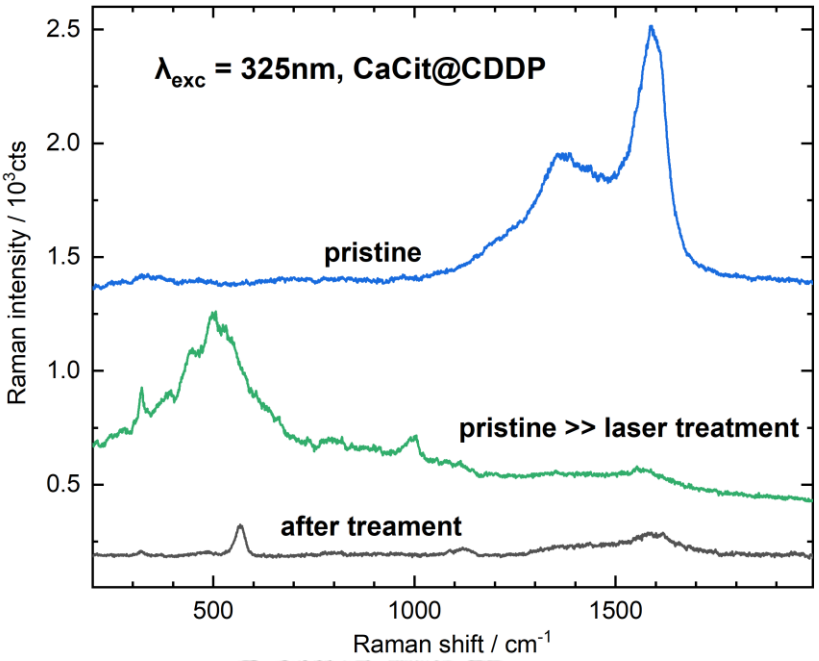


Figure 29. Raman spectra of CaCit@CDDP2 before and after treatment with UV (325 nm) laser.

The surface of nanoparticles was destroyed confirming *via* image from microscope as shown in figure 30. The black spot was evidently caused by UV laser. In this experiment, CaCit@CDDP nanoparticles were investigated that CDDP does not exist only in the core of CaCit nanoparticles. Next, the final product (CaCit@CDDP-B12) was also measured by Raman to prove that B12 was coordinated whether to modify the surface of nanoparticles.

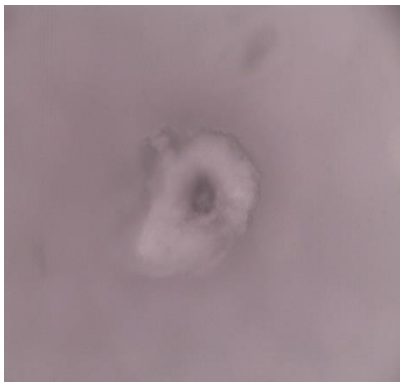


Figure 30. image of CaCit@CDDP2 after treatment with UV (325 nm) laser by microscope.

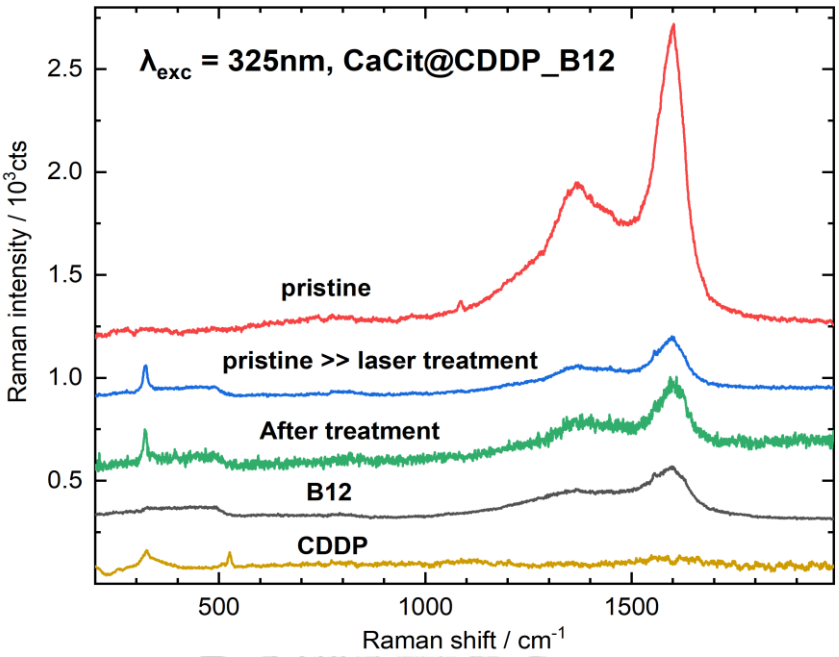


Figure 31. Raman spectra of CaCit@CDDP-B12 before and after treatment with UV (325 nm) laser.

The final product was analysed by Raman spectroscopy (fig. 31) with 450 μW of UV (325 nm) laser (red spectrum). As it has mentioned before (fig 25), an intense band at 1600 cm^{-1} is the signal of organic compound heated by UV laser. Additionally, it is also because B12 was fully absorbed UV (325 nm) laser. It indicated the band could be B12. Moreover, CDDP bands did not detect in this spectrum. Subsequently, 4.5 mW of UV laser was used. The band of CDDP has appeared as shown in blue spectrum (fig. 3.19). After the treatment by UV laser, the product was measured Raman with 450 μW. The CDDP band was also detected from a sample as shown in green spectrum. This experiment confirmed that CDDP was evidently encapsulated by CaCit nanoparticles. Afterward, a green (514 nm) laser was used in the next experiment for investigating B12.

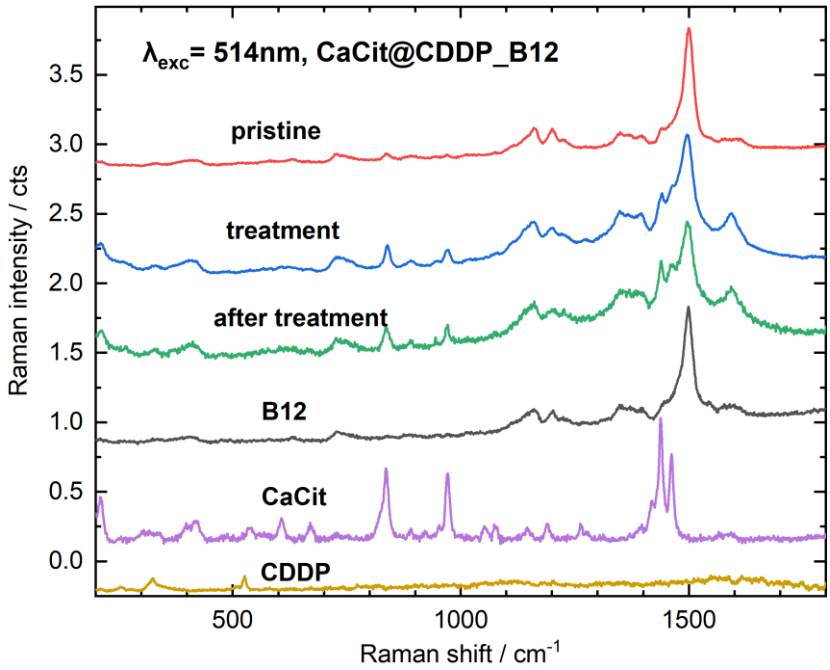


Figure 32. Raman spectra of CaCit@CDDP-B12 before and after treatment with UV (514 nm) laser.

The sample was started measuring raman with 85 μW of green (514 nm) laser (fig 32). First, a band (red spectrum) at 1500 cm^{-1} was obviously assigned to ring stretching modes of small nitrogen heterocycles from cyanocobalamin. Afterwards, increasing the power of green laser to 850 μW (blue spectrum) was perfectly hit carboxylate symmetric stretching mode from citrate ν_s(COO) at 1440 cm^{-1}. Then, the sample was continuously measured with 85 μW of green laser. The green spectrum showed that not only bands of carboxylate symmetric stretching had appeared but C=O stretching at 1613 cm^{-1} associated with citrate was also observed in this spectrum. It absolutely confirmed that CaCit nanoparticles were grafted by cyanocobalamin. However, the spectra in this experiment have not been revealed bands of CDDP. It is because of the power of green laser which could not be enough to excite vibration of ν(Pt–Cl) and ν(Pt–N) stretching.

3.2.3 Inductively coupled plasma - optical emission spectrometry (ICP-OES)

ICP-OES was used to characterise the percentages of encapsulations of CDDP and also percentages of grafted B12 by characteristic emission spectrum of platinum(II) and cobalt(II). The result was shown in the table 6. First, CaCit@CDDP were analyzed to measure Pt^{2+}. The calibration curve of Pt^{2+} was shown in figure 33. The concentrations of Pt^{2+} diluted by 100 times are approximately 4.72 ppm. It indicated that the actual concentrations of Pt^{2+} associated with CDDP are 472 ppm. The theoretical encapsulated concentrations of Pt^{2+} are 6500 ppm. Therefore, percentage of encapsulations of CDDP converted from Pt^{2+} are approximately 7.26%.

Table 6. Raw data from ICP-OES results.

$[Pt^{2+}]_{std}$ and $[Co^{2+}]_{std}$ (ppm)	Intensity of $[Pt^{2+}]$ (cts)	Intensity of $[Co^{2+}]$ (cts)
0.1	390.9	4542
1	1226	9104
3	2832	25860
5	4467	42910
7	6390	59050
9	7683	74100
CaCit@CDDP	4277	73.52
CaCit@CDDP-B12	14883	23553

Furthermore, the concentrations of Pt^{2+} in CaCit@CDDP-B12 were also calculated by calibration curve (fig 33) which are about 1750 ppm. It implied that % encapsulations of CDDP are approximately 14.96% (theoretical concentrations of Pt^{2+} are 11700 ppm) which is significantly increased from CaCit@CDDP in accordance with TGA results (fig. 3.10). This is because of Pt from B12-CDDP conjugates. Additionally, figure 3.21 has also shown the calibration curve of Co. The actual concentrations of Co^{2+} in CaCit@CDDP-B12 are approximately 271.4 ppm. It indicated that %grafted B12 are about 9.20% (theoretical concentrations of Co^{2+} are 2950 ppm).

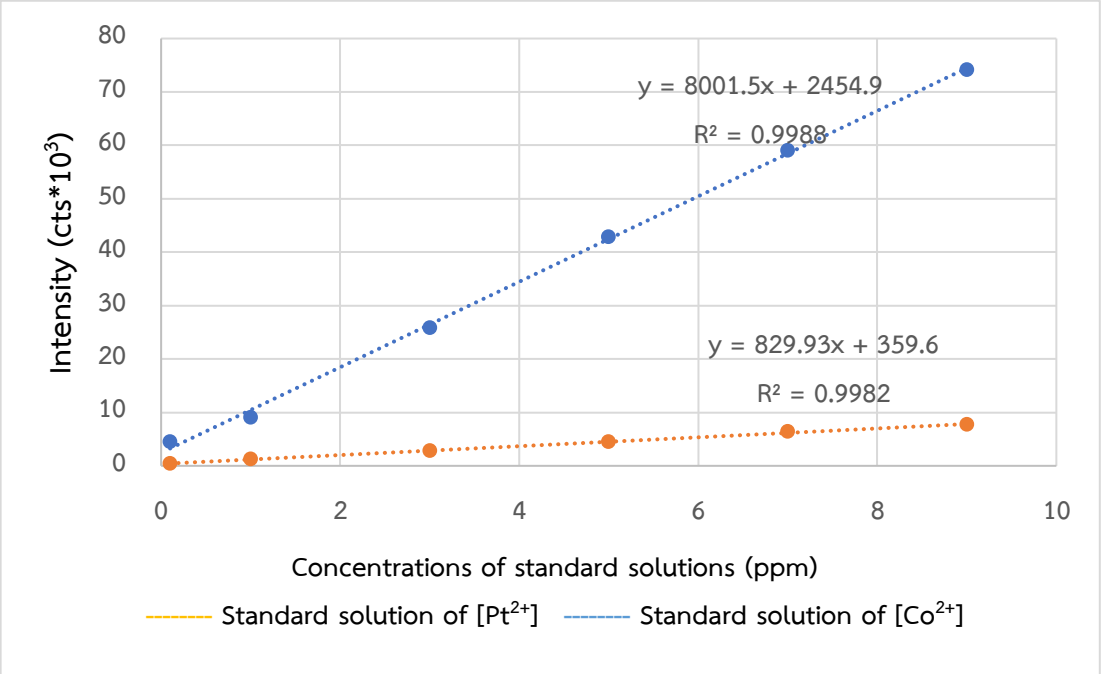


Figure 33. Calibration curve of $[Pt^{2+}]$ and $[Co^{2+}]$

3.3 Cytotoxicity study

The Human lung carcinoma epithelial cells (A549) were treated with CaCit@CDDP-B12 for 48 h to examine its effects on anticancer activity. Percentage of cell viability was also decreased by CDDP treatment (fig. 34) CaCit@CDDP-B12 significantly decreased the cell viability to lower than in cells treated with CDDP, whereas CaCit without drug had no effect on cell viability. These findings suggested that CaCit@CDDP-B12 effectively inhibited the cell viability of A549 cells. However, our result showed that CaCit@CDDP treatment was also no effect on cell viability in A549 cells.

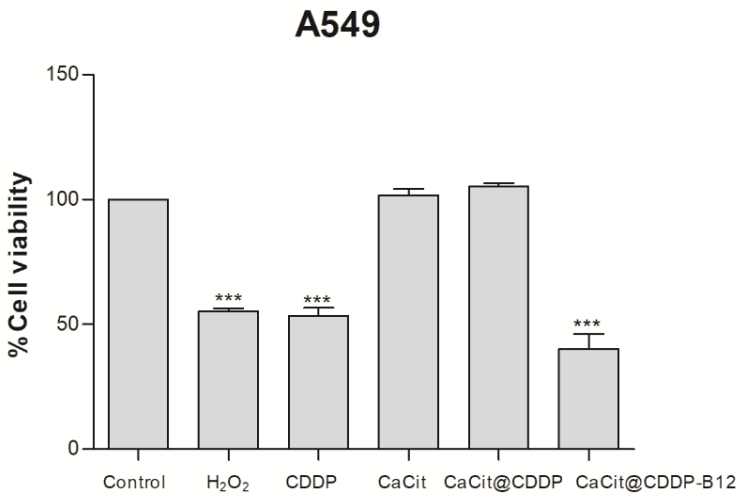


Figure 34. Effect of CaCit nanoparticles on A549 cell viability.

CHAPTER IV

CONCLUSION

CaCit@CDDP-B12 nanoparticles were successfully synthesized by 67% yield *via* chemical precipitation method. The obtained size of the spherical nanoparticles was approximately 553.0±65.0 nm. The percentages of the cisplatin encapsulations and grafted vitamin B12 were approximately 14.7% and 9.20%, respectively. Moreover, the interior architecture of the nanoparticles was executed *via* 2 steps. First, cisplatin was mixed with calcium citrate in the core of the nanoparticles. Second, CaCit@CDDP nanoparticles were evidently grafted on the surface by vitamin B12. Cytotoxicity study revealed that CaCit@CDDP-B12 effectively inhibited the cell viability of Human lung carcinoma epithelial cells (A549).

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