DEVELOPMENT AND CHARACTERIZATION OF DUAL MODALITY SYSTEM USING POLYMERIC NANOCARRIER

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Dedicated to my parents.

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ABSTRACT

Theranostic micelles and polymeric nanocarrier-based drug delivery system are two fields that have shown significant commitment in cancer treatment. Co-delivery of therapeutic and diagnostic agent in polymeric micelles for combination of therapy and diagnosis able to detect cancer cell in early stage, increase killing effect and suppress multidrug resistance (MDS) for better therapeutic effectiveness. Biocompatible and biodegradable polymeric nanocarriers generally have higher stability, sustained and controllable drug-release profiles and higher loading capacity for hydrophobic (poorly water soluble) drugs. Therefore, the aim of this study is to develop a dual modality micellar system using D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) as nanocarrier for co-delivery of docetaxel as a model chemotherapeutic drug and coumarin-6 as a model fluorescence imaging agent for simultaneous cancer imaging and therapy in early stage. The theranostic micelles were prepared by solvent casting method and characterize for their particle size, drug loading, drug encapsulation efficiency and *in vitro* drug release profile. This dual modality micellar system was successfully developed with average particle size of 79.59 nm in diameter and drug loading up to 15.46 µg/mg (encapsulation efficiency of 78.99%) and 9.83 µg/mg (encapsulation efficiency of 36.20%) for docetaxel and coumarin-6 respectively. Besides, the *in vitro* drug release profile of the micelles revealed a desired sustained and controlled drug release manner for both docetaxel and coumarin-6. In conclusion, the micelles size obtained is in favourable range for passive targeting through enhanced permeability and retention (EPR) effect and the drug loading and encapsulation efficiency attained are adequate for therapy and diagnosis purposes on cancer cells. The sustained drug release also beneficial in lower drug administration. This dual modality system is taking great advantages for tumour imaging and inhibition of tumour growth which is very important for early cancer detection, thereby having efficiency therapy on cancer cells.

ABSTRAK

Theranostic micelles dan sistem penyampaian ubat berasaskan pembawa polimerik bersaiz nano merupakan dua bidang yang membawa komitmen yang besar dalam rawatan kanser. Penghantaran serentak antara terapeutik dan diagnostic ejen dalam micelles polymeric demi kombinasi terapi dengan diagnosis membolehkan sel-sel kanser dikesan pada peringkat awal, meningkatkan keupayaan membunuh sel-sel kanser dan menindas rintangan pelbagai ubat untuk keberkesanan terapeutik yang lebih baik. Umumnya, pembawa polimerik bersaiz nano yang bioserasi dan bioterurai mempunyai profil pembebasan ubat yang lebih stabil, kekal, mudah dikawal dan kapasiti muatan yang lebih tinggi kepada ubat hidrofobik (kelarutan water rendah). Oleh itu, kajian ini bermatlamat untuk mewujudkan sistem misel dwi-modaliti dengan menggunakan D-α-tokoferol polyethylene glycol 1000 succinate (TPGS) sebagai pembawa nano dalam penghantaran serentak docetaxel yang merupakan model ubat kemoterapeutik dan coumarin-6 yang merupakan model ejen pengimejan pendarfluor untuk pemgimejan kanser dan terapi serentak pada peringkat awal. *Theranostic micelles* disediakan dengan cara pemutus pelarut dan dicirikan berdasarkan saiz partikel, muatan ubat, kecekapan pengkapsulan ubat dan profil pembebasa ubat *in vitro*. Sistem misel dwi-modaliti ini berjaya diwujudkan dalam purata saiz partikel 79.59 nm dalam diameter dan muatan ubat sebanyak 15.46 µg/mg (kecekapan pengkapsulan sebanyak 78.99%) dan 9.83 µg/mg (kecekapan pengkapsulan sebanyak 36.20%) untuk docetaxel dan coumarin-6 masing-masing. Selain itu, profil pembebasan ubat *in vitro* micelles mendedahkan cara pembebasan ubat yang mendorong, kekal dan terkawal kepada docetaxel dan coumarin-6. Kesimpulannya, saiz micelles didapati berada dalam julat yang meluluskan untuk sasaran pasif melalui kebolehtelapan yang ditingkatkan dan kesan pengekalan (EPR) dan juga muatan ubat serta pencapaian kecekapan pengkapsulan adalah mencukupi untuk terapi dan kegunaan diagnosis untuk sel-sel kanser. Pembebasan ubat yang kekal membawa manfaat dalam mengurangkan pengambilan ubat. Sistem dwi-modaliti ini membawa kebaikan kepada pengimejan tumor dan perencatan pertumbuhan tumor yang memainkan peranan penting kepada pengesanan kanser peringkat awal, dengan itu juga membawa keberkesanan dalam terapi sel-sel kanser.

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LIST OF ABBREVIATIONS

ACS	American Cancer Society
ADME	Adhesion, distribution, metabolism and excretion
BCS	Breast conservation surgery
BCT	Breast conservation therapy
CMC	Critical micelle concentration
DCM	Dichloromethane
DLS	Dynamic light scattering
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention
ER	Estrogen receptor
FDA	Food and Drug Administration
HER2	Epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
MDR	Multidrug resistance
PBS	Phosphate buffered saline
pCR	pathologic complete response
PEG	Polyethylene glycol
P-gp	P-gylcoproteins
PLGA-PEG	poly(D,L-lactic-co-glycolic acid)-block-poly(ethylene glycol)
PR	Progesterone receptor
RES	Reticuloendothelial system
RME	Receptor-mediated endocytosis
TNBC	Triple negative breast cancer
TPC6	Coumarin-6 loaded TPGS micelle

TPD	Docetaxel loaded TPGS micelle
TPDC6	Docetaxel-coumarin-6-loaded vitamin E TPGS micelles
TPGS	D-α-tocopheryl polyethylene glycol 1000 succinate
UV/VIS	Ultraviolet/visible

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cancer which is commonly characterized by uncontrolled cell growth caused by up regulation of oncogenes or down regulation of tumour suppressor genes and angiogenesis is one of the leading causes of death worldwide (Siegel et al., 2015). Molecular subtypes of breast cancer included luminal A, luminal B, basal-like breast cancer, triple negative breast cancer (TNBC) and epidermal growth factor receptor 2 (HER2) positive breast cancer (Nounou et al., 2015). Breast cancer generally classified based on tumour markers of progesterone receptor (PR), estrogen receptor (ER) and HER2 (Parise and Caggiano, 2014). However, TNBC lack of these three receptor proteins (PR^- / ER^- / $HER2^-$). TNBC subgroup comprise approximately 15% of all types of breast cancer with a significantly higher percentage found in younger women. In a cohort study of 1601 breast cancer patients, mean age of women diagnosed with TNBC was 53 years whereas patients with other subtypes was 58 years (Cleator et al., 2007; Bartsch et al., 2010). The incidence of TNBC cases in Malaysia is approximately 17%, a rate similar to that found in Western studies (Dean and Rhodes, 2014).

Treatment options currently available for TNBC are limited to standard cytotoxicity chemotherapy such as paclitaxel due to the lack of therapeutic target required to effectively deliver drugs to tumour with minimal by-effect on healthy cells and multidrug resistance (MDR) exhibited by TNBC tumour that prevents delivery of therapeutic agents into tumours (Pal et al., 2012; O'Reilly et al., 2015). However, nanomedicine offers effective solutions to overcome the problems encountered by conventional chemotherapy. Nanomedicine able to deliver therapeutic agents into the targeted cells via receptor-mediated endocytosis (RME) in addition nanocarriers able to

conquer MDR by providing protection to therapeutic agents and prevent elimination of P-glycoproteins (P-gp) (Kutty, 2015). Issues with conventional chemotherapy such as drug resistance, formulation and pharmacokinetics (controlled drug release) may be solved with the assist of nanomedicine.

Patients with TNBC are usually detected at the late stage, have an aggressive tumour type due to high rate of local recurrences and systematic metastases and a poorer prognosis compared to patients with non-TNBC (Cao et al., 2010). In addition, the mean time to local and distant recurrence were shorter in patients with TNBC than other cancers which were 2.8 versus 4.2 years and 2.6 versus 5.0 years respectively (Dent et al., 2007). These patients were significantly more likely to have visceral metastases, which might contribute to their poorer prognosis. Hence, there is a clear demand to examine nanotheranostics for sensitive diagnosis at its earliest stage and effective therapy act on TNBC tumour by therapeutic agent.

The aim of this study is to develop and characterize a novel theranostic micelle system for TNBC treatment. Docetaxel is chosen as the model anticancer agent or therapeutic agent whereas coumarin-6 is chosen as the diagnostics or imaging agent and both are encapsulate by D-α-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS/TPGS) which act as the nanocarrier to deliver the chemotherapeutic agent and diagnostic agent into TNBC cells. Docetaxel leads to mitotic arrest in the G2/M phase of cell cycle by binding to microtubules and causes cell death (Kutty, 2015). Coumarin-6 with fluorescence property is able to act as fluorescent probe for applications such as biomarker and bioimaging to visualize morphological details and monitor biomolecule in living systems (Chen et al., 2013). Vitamin E consist of both lipophilic alkyl tails and hydrophilic polar heads was used to synthesize the polymeric micelle which above its critical micelle concentration (CMC) of 0.02 wt%. It is hypothesized that the theranostic micelle system will be able to synthesize with higher drug loading within the expected micelle size.

1.2 Motivation

Coumain-6 with great fluorescence imaging capacity can serve as a powerful and promising candidate for bioimaging probe which required for early diagnosis of cancer in order to be treated efficiently with therapeutic drugs. There is higher chance to be treated efficiently if cancer is able to diagnose at an early stage as treatment becomes more difficult if the cancer cell has spread and generally lower chance of surviving.

The co-delivery of both chemotherapeutic agent and diagnosis agent in a dual modality micelle system form a theranostic nanoparticle that can be used in the diagnosis and therapy on cancer cell in early stage which can control local recurrences and prevent distant metastases.

1.3 Problem statement

TNBC is one of the most aggressive cancer due to its higher rate of local recurrences and distant metastases. TNBC represents a challenge for clinicians due to its poorer prognosis in comparison to other breast cancer subtypes and fewer treatment options, with a lack of targeted therapy for effective delivery of chemotherapeutic agents into metastasised TNBC cells (Wahba and El-Hadaad, 2015). Cancerous cells tend to develop MDS which is a major factor in the failure of many forms of chemotherapy (Kutty, 2015). Therefore, we postulated a novel polymer-based nanocarrier for the co-delivery of fluorescence imaging agent (coumarin-6) and chemotherapeutic drug (docetaxel) in order to develop the tumour-imaging for early cancer detection, thereby having efficiency therapy in early stage in addition inhibit MDS and reducing the rate of recurrence and metastasis.

1.4 Objectives

The following are the objectives of this research:

1) To develop and characterize a novel dual modality system using polymeric nanocarrier for the diagnosis and therapy of cancer disease.
2) To study the drug release profile of the micelle system.

1.5 Scopes of study

The following are the scopes of this research:

1) To synthesize the dual modality micelle system of docetaxel-coumarin-6-loaded vitamin E TPGS micelles (TPDC6).
2) To optimize the micelle system to achieve desired drug loading and encapsulation efficiency.
3) To characterize the micelle system to achieve desired particle size and precise drug release in neutral pH 7.4.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a class of diseases characterized by uncontrolled growth of malignant cells and form cancerous tumours. Malignant tumours may invade surrounding tissues or organs in addition undergo distant metastasis through the blood or the lymphatic system (Kutty, 2015). There are more than 100 different types of cancer which is classified by the type of cell that is originally affected. In Malaysia, cancer is now the fourth leading cause of death among medically certified deaths (Lim, 2002). The incidence of cancer in Malaysia increased from 32,000 new cases in 2008 to 37,400 in 2012. This number is expected to rise to 56,932 by 2025 if no action is taken (“Rise in cancer deaths in M’sia,’’2014).

2.1.1 Types of breast cancer

The National Cancer Registry (NCR) 2003-2005 reported an age-standardised rate (ASR) of 47.3 per 100,000 in Malaysia. The incidence is highest in Chinese (59.9 per 100,000) followed by Indians (54.2 per 100,000) and Malays (34.9 per 100,000), The International Agency for Research in Cancer (GLOBOCAN) 2012 estimated the ASR of breast cancer in Malaysia as 38.7 per 100,000 with 5410 new cases in 2012 (Yip et al., 2014).

Breast cancers are distinguished by up to 21 distinct histological subtypes and at least four different molecular subtypes which are identified based on the type of receptors present at the molecular level: progesterone receptor (PR+/PR-), estrogen receptor

(ER+/ER-) and epidermal growth factor receptor 2 (HER2+/HER2-), a growth promoting protein (Kutty, 2015). The four main molecular subtypes are luminal A (PR+/ER+/HER2-), luminal B (PR+/ER+/HER2+), HER2-enriched (PR-/ER-/HER2+) and triple negative (PR-/ER-/HER2-) (American Cancer Society [ACS], 2015).

The classification of tumours provide important information on the action of tumours and also in the selection of therapeutic regimen for patients. For PR or ER positive tumours, hormonal therapy (also called endocrine therapy) is used to lower ER levels or to constrain growth of breast cancer cell by hindering the effect of ER (ACS, 2015). Tamoxifen and aromatase inhibitors such as letrozole, anastrozole, and exemestane are clinically used to prevent recurrence after surgery (ACS, 2015; Kutty, 2015). For HER2 over-expressing tumours, monoclonal antibodies such as Trastuzumab (Herceptin) and Pertuzumab (Perjeta) are used to bind HER2 receptors for inhibiting cell proliferation (ACS, 2015). However, triple negative patients do not benefit from existing hormonal or trastuzumab-based therapies because of the lack of target receptors such as PR, ER and HER2, hence, prognosis remains poor and there is no targeted therapy developed to prevent recurrence and metastasis of tumours (Wahba & El-Hadaad, 2015).

2.1.2 Triple negative breast cancer (TNBC)

TNBC is a particularly difficult type of cancer to cure and biologically aggressive disease with high proliferation rate, high risk of early recurrence and distant metastases compared with other types of breast cancer. It is characterized by the lack of overexpression of all three receptors: ER receptor, PR receptor and HER2 receptor (Conte & Guarneri, 2009).

TNBC does not respond well to hormone therapy which disrupts hormone's ability to assist cancer growth or medications that block HER2 receptors because TNBC cells lack of overexpression of ER, PR and HER2 genes. Instead of hormone therapy, treating TNBC often involves chemotherapy, surgery and radiation. TNBC can often be treated successfully if it is detect earlier. But in general, survival rates tend to be lower with TNBC compared to non-TNBC breast cancer. Moreover, TNBC is more likely than

other types of breast cancer to recurrence especially in the first few years after treatment (Godman, 2014).

The identification of the molecular subtypes is beneficial in terms of predicting prognosis, understanding the biological characteristics and guiding treatment recommendations. Six TNBC subtypes were identified by Cluster analysis based on the gene expression profile, including two basal-like, a mesenchymal, a luminal androgen receptor, an immunomodulatory and a mesenchymal stem-like subtype. TNBC represents the majority (80%) of cancers within the basal-like subtype. TNBC classified as basal-like phenotype due to the expression of basal's markers such as basal cytokeratin (5, 6, 14 and 17), vimentin, epidermal growth factor receptor (EGFR), mutated BRCA1/2 and deleted p53 (Kutty, 2015). TNBC with basal-like subtypes are more aggressive tumours and well known to predict poor outcome (Bartsch et al., 2010).

Patients with TNBC were more likely to have died than patients with non-TNBC. The median time to death was 4.2 years for patients with TNBC compared with 6 years for patients with non-TNBC. Patients with TNBC and non-TNBC had similar rates of local recurrence but a higher proportion of patients with TNBC experienced distant recurrence. Furthermore, the mean time to local and distant recurrence were shorter in patients with TNBC than non-TNBC which were 2.8 years versus 4.2 years and 2.6 years versus 5.0 years respectively (Dent et al., 2007). These patients were significantly more likely to have visceral metastases, which might contribute to their poorer prognosis.

2.2 Current cancer therapies for TNBC

Patients with TNBC were not sensitive to the trastuzumab or hormonal-based therapy because of the lack of target receptors such as ER, PR and HER2. Therefore, surgery, radiotherapy and chemotherapy, in combination or individually, appear to be the only accessible modalities for TNBC (Wahba & El-Hadaad, 2015; Kutty, 2015). There are two basic types of surgery which are breast conservation therapy (BCT) and mastectomy. BCT involves removal of tumour followed by radiation therapy whereas mastectomy involves removal of the entire breast. However, surgery has few drawbacks which are wound infection, not feasible for patients with medical history, trigger

metastasis and lastly the adjuvant radiotherapy and chemotherapy may affect the dividing cells of normal tissue which lead to side effect (Kutty, 2015). Besides, there is currently no specific systemic regimen recommended for the treatment of TNBC (Cleator et al., 2007). However, certain studies have recognized some receptors as targets for new therapeutic drugs.

2.2.1 Surgery

The conventional treatment for patients with TNBC is surgical excision of the breast tumour mass by breast conserving surgery (BCS) followed by radiation therapy and mastectomy (Eiermann & Vallis, 2012). Surgical decision making likely depends more on traditional clinicopathological variables and patient priority. Local recurrence rate after BCS is not high in TNBC as those of non-TNBC so they remain appropriate choices for breast conservation (Wahba & El-Hadaad, 2015). Parker et al. (2010) proposed that the 5-year disease-free survival rates for the mastectomy and BCT groups were 57% and 68% respectively whereas the 5-year overall survival was better for the BCT than for the mastectomy group (89% versus 69%) (Eiermann & Vallis, 2012). Freedmann et al. (2009) reported that insignificant difference was witnessed between TNBC and non-TNBC patients in terms of breast recurrence free survival at five years after BCT (Kutty, 2015). In contrary, Nguyen et al. (2008) has stated that a greater local recurrence rate at five years was observed for the basal-like subtype compared with other subtypes.

2.2.2 Radiotherapy

Radiotherapy is using high energy radiation to control or destroy cancer cells. Radiotherapy is given in TNBC as demonstrated in other breast cancer subtypes following BCS or mastectomy and combined with chemotherapy. BCS followed by radiotherapy in early stage may not be equivalent to mastectomy as in non-TNBC due to TNBC are locally aggressive and rapidly growing cancers. However, Abdulkarim et al. (2011) reported that women with TNBC harbour a pathogenic mutation in the BRCA1 gene and tumours lacking practical BRCA1 are potentially highly radiosensitive (Wahba & El-

Hadaad, 2015). Abdulkarim et al. (2011) and Wang et al. (2013) reported that patients with TNBC tumours who received radiotherapy after surgery had reduced risk of locoregional recurrence and increased overall survival in comparison to those that did not receive radiaotherapy (Steward et al., 2014). Kyndi et al. (2008) proposed that radiotherapy reduced local recurrence after mastectomy in TNBC. Still, no enhancement in overall survival was examined for radiation in TNBC (Kutty, 2015).

2.2.3 Chemotherapy

Several reports recommend that TNBC respond to chemotherapy better than other subtypes of breast cancer although prognosis remains poor (Ismail-Khan & Bui, 2010). This is because more violent course in metastatic setting and shortened disease-free interval in adjuvant and neoadjuvant setting. There are no criterion chemotherapy regimens for TNBC. The therapeutic approaches for the management of TNBC are cell proliferation like (anthracycline containing treatment), targeting DNA repair complex like (taxanes and platinum compounds), P53 like (taxanes), and targeted therapy. Also several neoadjuvant studies have sought to determine the additive benefit of incorporating novel chemotherapeutics with standard chemotherapy like anthracycline, taxanes, antimetabolites, platinum agents and novel microtubule stabilizing agents (Wahba & El-Hadaad, 2015). Basal-like and HER2+/ER- subtypes are more sensitive to anthracycline-based neoadjuvant chemotherapy than luminal breast cancers (Carey et al., 2007).

Patients with TNBC have improved response to neoadjuvant chemotherapy (rate of pathologic complete response [pCR]) compared with non-TNBC, and those with pCR have excellent survival. However, mostly in the first 3 years, patients with residual disease after neoadjuvant chemotherapy have significantly inferior survival if they have TNBC compared with those non-TNBC (Liedtke et al., 2008). Also, the neoadjuvant setting provides an opportunity to determine *in vivo* tumour responses to chemotherapy (Gluz et al., 2009).

In the context of metastases, TNBC patients have relatively shorter median survival due to higher rates of visceral metastases and thus have limited duration of response to successive lines of chemotherapy. Therefore, prediction of response to

specific chemotherapy by identification of molecular biomarkers is required to further improve treatment strategies with the current chemotherapy options and future combination with targeted therapies (Wahba & El-Hadaad, 2015).

Over the past few years, the advantages of taxanes in adjuvant therapy of TNBC has been recognised. The first trial that established the advantage of paclitaxel in TNBC has resulted in 17% reduction in the risk of relapse and 18% reduction in the risk of death with an enhancement in 5-year disease-free survival and overall survival. This concluded that addition of taxanes in the adjuvant setting are important in TNBC (Wahba & El-Hadaad, 2015). However, there is presently no particular standard chemotherapy to deal with patients with relapsed metastatic TNBC (Andre & Zielinski, 2012).

2.2.4 Combination therapy

Combination anticancer treatment has long been implemented in clinics for better therapeutic effectiveness. Combination therapy may compromise improved outcomes in term of progression–free survival. For instance, a significantly prolonged progression-free survival for the combination regimen (6.0 months vs 4.0 months) was proved by a study of vinorelbine plus gemcitabine versus vinorelbine alone. In addition, for patients with violent triple-negative disease given their poor prognosis, especially when disease has metastasised, combination chemotherapy of cytotoxic and targeted agents may still be of particular clinical relevance (Conte & Guarneri, 2009).

Antharactclines-based combination chemotherapy has revealed better anticancer action than anthhracyclines only. In a study, doxorubicin has attained response rate of 60%-70% in combination whereas 40%-50% as single agent. Joensuu et al. (1998) stated improved response rate of 55% in patients treated with epirubicin with cyclophosphamide and fluorouracil (FEC) than 48% in patients treated with epirubicin only. However, most of the epirubicin-treated patients (59%) experienced slight or no hair loss while majority of FEC-treated patients (80%) suffered from total hair loss (LEE & Nan, 2012). Moreover, cardiotoxicity that lead to development of ppotentially fatal congestive heart failure limited the clinical advantages of anthracyclines (Livi et al., 2009).

Combination therapy of taxanes and anthracyclines improved time to progression and overall survival compared to anthracycline-based combination therapy. Although greater toxicity appears from taxane containing regimen (74%) than the anthracycline regimen (63%), a substantially greater therapeutic benefit may be overcome the overall added toxicity of an anthracycline/taxane combination. Taxane with nonanthracycline combinations which is another extremely effective regimen is particularly beneficial in patients with promptly progressive visceral metastases, who were previously treated with an anthracycline. Albain et al. (2008) reported the combination of gemcitabine and paclitaxel regimen has better response rate (41% versus 26%) and longer time to progression (6 months versus 4 months) compared to paclitaxel alone. However, toxicity of this combination was higher with increased neutropenia, fatigue and neuropathy (Lee & Nan, 2012).

2.3 Limitations of conventional chemotherapy in TNBC

There are some factors involved in determining the effectiveness of a chemotherapeutic effort. One of the factors is dosage form. Adjuvants which may cause side effects and life-threatening have to be used for many anticancer drugs due to highly hydrophobic properties which are not soluble in water. For instance, paclitaxel uses an adjuvant consisting of Cremophor EL which causes serious side effects, including hypersensitivity reactions, neurotoxicity and cardiotoxicity (Feng & Chien, 2003).

Drug pharmacokinetics is an important factor too. The objective of chemotherapy is to deliver high-efficacy drugs at the right time to the right location at the right concentration over the right length of time. This pharmacokinetic sweet spot is hard to accomplish due to the distinctly different transient states of drugs in adhesion, distribution, metabolism and excretion (ADME) (Kutty et al., 2014).

In addition, systemic drug toxicity is another factor that need to be concerned. Free anticancer drug molecules are toxic to healthy cells with rapid turnover, such as bone marrow cells and intestinal epithelium cells. Liver and kidney, which have important roles in drug metabolism and excretion, may be damaged by the chemotherapy. It would be

ideal if the chemotherapeutic drugs could only exert their actions on the cancerous cells and leave the normal cells unaffected (Feng & Chien, 2003).

Moreover, cancer cells tend to develop drug resistance, for example MDR, over the prolonged course of treatment, which can eventually lead to failure of chemotherapy. There are three major categories of drug resistance: pharmacokinetic resistance due to the lower than efficacious dose of drug in the tumours, kinetic resistance due to the occurrence of only a small portion of cells in a susceptible state, and genetic resistance due to the biochemical resistance of the tumours cells against the drug (Feng & Chien, 2003; Kutty et al., 2014).

2.4 Nanomedicine

Nanomedicine refers to the practice of nanotechnology in medical applications. Nanomedicine offers several potential advantages over conventional treatment for cancers, including the early detection of cancers and cancer treatment, active and passive disease targeting, increased biocompatibility, and multifunctionality encompassing both imaging and therapeutic capabilities, allowing for simultaneous disease treatment and monitoring (Wang et al., 2013). Firstly, the desired size (e.g., 1 nm-100 nm in diameter), surface charge and surface modification (e.g., by polyethylene glycol, PEG or TPGS) of the nanoparticles which can reduce the opsonisation and minimizes the clearance by the reticuloendothelial system (RES) to realize a sustained and controlled drug release of sufficiently blood circulation times and improved pharmacokinetic properties (Kutty et al., 2014; Nie, 2010).

Secondly, nanomaterials can also be functionalized with biomolecules, enabling them to target delivery of the therapeutic agent to specific organelles within certain tissues or even the entire cells and enhance internalization of nanoparticles into the tumour via endocytosis, which avoid recognition by the P-gp. Thirdly, nanoparticles can be utilized for bioimaging due to the novel physical properties, such as optical properties from quantum dots. Furthermore, nanoparticles formulations, through passive or active targeting, allow the therapeutic drugs to accumulate and release in tumours via the

enhanced permeability and retention (EPR) effect, thus significantly reducing systematic toxicities (Wang et al., 2013; Kutty et al., 2014).

Lastly, nanomedicine can further improve treatment efficacy by co-delivery of two or more therapeutic agents for combined therapy. Nanomedicine may provide a practical solution to TNBC treatment due to the possibilities of high drug loading and encapsulation efficiency, high transportation efficiency across the cell membrane, long half-life in blood circulation, as well as desired pharmacokinetics (Kutty et al., 2014).

2.5 Nanomedicine for treatment of TNBC

Several nanocarriers such as prodrugs, micelles, liposomes and biodegradable polymeric nanoparticles that used to deliver therapeutic agents have been verified to have considerable effects compared with free drugs. According to Taurin et al. (2013), a micellar system has been synthesized by using styrene-co-maleic acid (SMA) to deliver hydrophobiccurcumin (3,5-bis(3,4,5-trimethoxybenzylidene)1-methypiperidine-4-one, RL71) for TNBC treatment. As a result of higher cellular uptake via endocytosis and slow release profile, the micellar formulation led to a higher cytotoxicity compared with free drugs. The finding was further supported *in vivo*, where the uptake into tumour site enhanced by the longer circulation of micelles in the blood system and eventually led to a higher cytotoxicity (Kutty et al., 2014). Furthermore, Johnstone et al. (2013) developed a polymeric nanoparticles of poly(D,L-lactic-co-glycolic acid)-block-poly(ethylene glycol) (PLGA-PEG) that stabilized with poly(vinyl alcohol) (PVA) to encapsulate the hydrophilic platinum (IV) prodrug mitaplatin via the water/oil/water double emulsion method. The results presented that same degree of tumour inhibition can be achieved by treatment using PLGA-PEG nanoparticles formulation with lower dosage was compared with the free drug (Johnstone et al. 2013).

Due to absence of targeted therapy of conventional drugs and its resistance to most chemotherapeutic drugs, the treatment for metastatic TNBC patients becomes a challenge. Kutty and Feng developed a system of cetuximab-conjugated micelles of TPGS for targeted delivery of docetaxel as a model anticancer drug for treatment of TNBC. Cetuximab is a human chimeric monoclonal antibody, which targets the epidermal growth

factor receptor (EGFR) which overexpressed in TNBCs as many as 2×10^6 compared with normal cells and prevents the binding of EGFR and activation of the EGFR signalling pathway. In the *in vitro* therapeutic effects on fibroblast cells (NIH 3T3), positive type breast cancer cells (MCF7 and SK-BR-3 of ER/PR, and HER2 overexpressed, respectively) and TNBC cells of all three formulations of docetaxel, that is, the free drug (Taxotere), the docetaxel-loaded Vitamin E TPGS micelles and the cetuximab-conjugated docetaxel-encapsulated micelles of Vitamin E TPGS, were investigated. The results showed that the presence of cetuximab in the micellar formulation promotes the uptake of nanoparticles via endocytosis and hence effectively kill all the three types of TNBC (Kutty & Feng, 2013). Besides chemotherapy, TNBC exhibits a high sensitivity to nanoparticle-based photothermal therapy but resistant to conventional hyperthermia therapy (Kutty et al., 2014).

2.6 Coumarin-6

Coumarin-6 is an orange needle crystal which can dissolve in methanol, ethanol, ethylene glycol and benzene ethanol. Their solution was green fluorescence which is very beneficial in nanomedicine. According to Makwana et al. (2011), coumarin-6 was used as fluorescent probes to monitor protein aggregation in their research for the first time. Interaction between hydrophobic patches causes protein aggregation in majority cases and they decided to examine the suitability of coumarin-6 as fluorescent probe to monitor the aggregation of bovine carbonic anhydrase (BCA) and a-synuclein. From their research, it is found that coumarin-6 is able to recognize and discriminate between amorphous and fibrillary type of protein aggregates (Makwana et al., 2011). Furthermore, a recent study showed that nanoparticles with green fluorescent dye (coumarin-6), blue fluorescent dye (perylene) and orange fluorescent dye (Nile red) have the potentials in bioimaging and sensing applications (Cicoira and Santato, 2007). Since coumarin-6's fluorescence increases with decrease in pH, it has been reported to be used as the pH sensitive probe and it is useful in the detection of cancerous cell as the environment surrounding the cancerous cell is in acidic condition. Coumarin-6 has also been utilized as a fluorescent lipophilic probe for studies of emulsion uptake in cancer cell lines (Makwana et al., 2011).

Fluorescent probes provide information relating the quantity and localization of the molecules of interest without the need of genetic engineering of the sample make it become an essential tool in modern biology. Fluorescence imaging has many benefits such as it enables non-invasive, highly sensitive and safe detection using readily available instruments compared to other technologies such as MRI, ESR, radioisotope labelling and electrochemical detection. Most recently, probes based on conjugated polymers and nanoparticles have been presented and some of them have already been applied *in vivo* imaging (Teraj and Nagano, 2008).

Coumarin-6 is traditionally used as a laser or fluorescent dye to facilitate the traceability of drug delivery system in vitro, visualization and quantitative analysis of cellular uptake of nanoparticles and is illustrated as tool to gain insight by fluorescence spectroscopy due to its highly fluorescent nature (Finke et al., 2014). According to Aboutaleb and Dinarvand (2012) study, coumarin-6 was selected as the fluorescent dye for drug delivery to brain because of limited leak from nanoparticles due to its high lipophilicity and high sensitivity of detection by fluorescence. Besides, it has the advantages of poor penetration into the brain and its elevated levels in brain can be considered as the ability of nanoparticles penetrate into the brain. Moreover, it is frequently used as a model hydrophobic drug for studies involving tracking of localized delivery and controlled drug release. The solubility of coumarin-6 in water is 0.25 µg/ml, which contributes it as an excellent model for hydrophobic drugs such as docetaxel, paclitaxel, everolimus, and others. In addition, it is in bright fluorescence yellow, thus it is easy to observe the drug delivery.

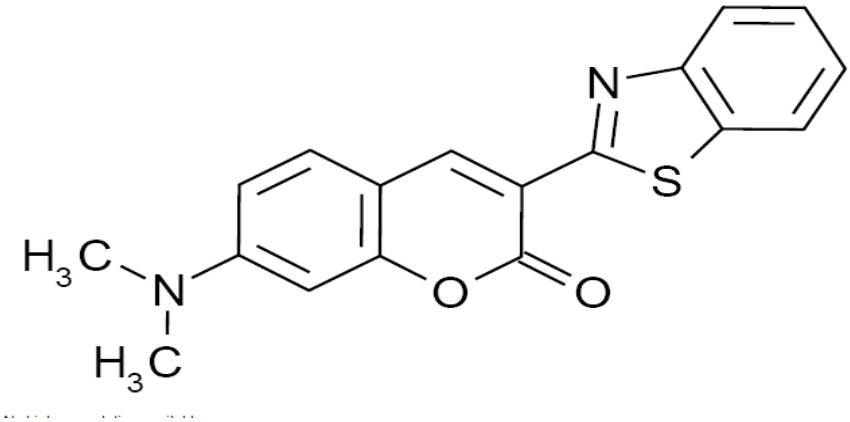


Figure 2.1: Molecular structure of coumarin-6 (Kristoffersen et al., 2013)

2.7 Docetaxel

Docetaxel (Taxotere) is one of the taxane type drugs that approved for clinical use by the Food and Drug Administration (FDA) for the treatment of several cancers such as breast, non-small cell lung cancer, prostate, stomach, head and neck cancers. They are hydrophobic anticancer agents that cause mitotic arrest in G2/M phase of the cell cycle by binding to microtubules which eventually results in the cell dead. Based on specific cancer type and stage, docetaxel can be used as a single drug or in combination with other chemotherapeutic drugs. Taxanes are formerly isolated from natural source of plants of genus Taxus. As a result of limited availability of yew trees which causes it is not economically practical to synthesize paclitaxel. Therefore, the use of semi-synthetic analogues is one of the potential solutions. Docetaxel which is a new generation semi-synthetic analogue that can be used as an alternative to paclitaxel (Kutty, 2015).

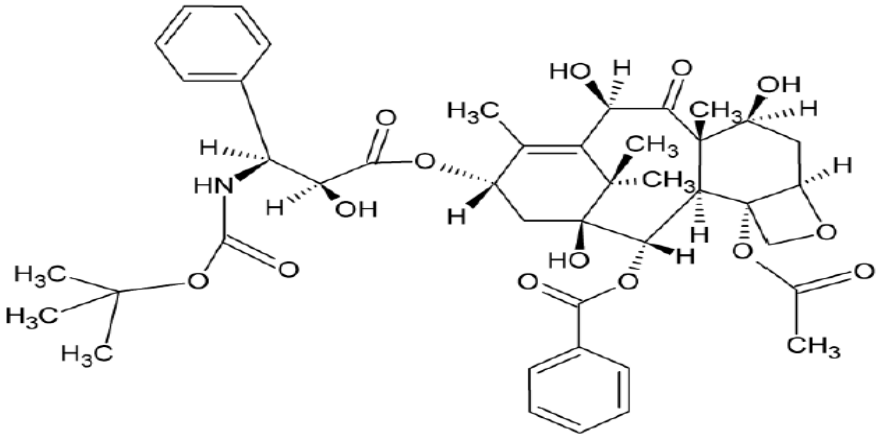


Figure 2.2: Molecular structure of docetaxel (Kutty, 2015)

2.8 Polymeric micelles as a drug carrier

Polymeric micelles with several desirable features which synthesized from amphiphilic di- or tri-block copolymers have gained prevalent attention in nanomedicine research (Kutty, 2015). Generally, the micelle self-assembles with the hydrophilic blocks to form the micelle shell and with hydrophobic blocks to form the core of the micelle. Micelle-based nanocarriers are biocompatible and physiologically stable due to their low CMC (Kang et al., 2015).

Polymeric micelles have numerous strong benefits as a drug carrier for chemotherapeutic drugs in cancer treatment. Hydrophobic agents which are poorly water soluble are recognised to be related with problems in therapeutic applications such as drug aggregation-related complications, bioavailability and poor absorption. On the other hand, many anticancer drugs are poor solubility in water. The hydrophobic core of polymeric micelle can solve this problem by acting as a reservoir for the poorly soluble hydrophobic drugs (Ayre et al., 2013; Kang et al., 2015).

Moreover, drugs should possess hydrophobicity to penetrate a cell membrane and acquire adequate affinity toward the target receptor. Micelle able to overcome this problem with the amphiphilic copolymers which are used to encapsulate poorly water-soluble anticancer drugs in inner hydrophobic core and outer shell of hydrophilic block of copolymer which diminishes the interactions of drug with the external aqueous environment keeping them stable. In addition, physicochemical properties of polymeric micelle for tumour targeting by EPR effect which is a passive targeting mechanism is a great advantages in chemotherapy (Ayre et al., 2013).

Polymeric micelles can be engineered by addition of pH-sensitive moieties and ligand coupling such as folate, sugars, peptides and transferrin to enhance the active targeting-ability of the carrier. Pharmaceutical drug carriers carrying drug in plasma should possess properties such as small size, prolonged circulation, biodegradability, high loading capacity and accumulation in appropriate site in the body. All these properties are mostly executed by polymeric micelles which make them to be an ideal drug carrier for anticancer drugs and targeting the cancerous cells (Ayre et al., 2013; Kang et al., 2015).

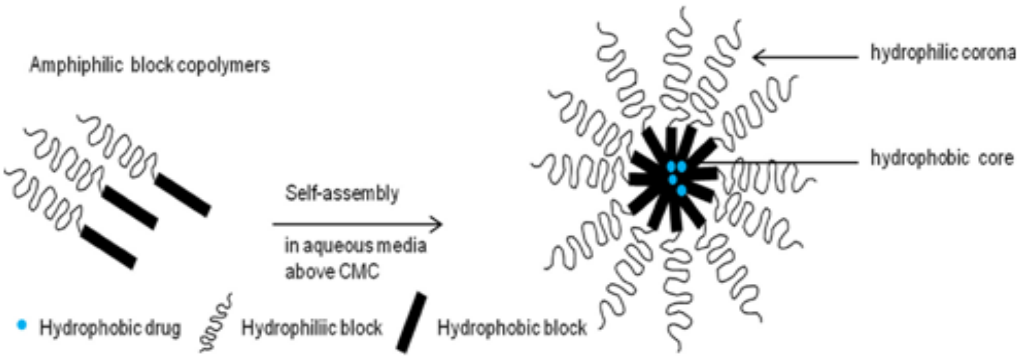


Figure 2.3: Illustration of micelle formation (Jhaveri & Torchilin, 2014)

2.9 Vitamin E TPGS based nanocarrier

D-α-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS or simply TPGS) is a white or slightly yellowish, waxy solid with melting point of 37˚C-41˚C and it is water-soluble (Kutty, 2015). TPGS is synthesized by esterification of D-α-tocopheryl acid succinate and PEG 1000. Recent years, TPGS has obtained increasing attention for pharmaceutical application, as a biomaterial in developing numerous drug delivery systems such as micelles, prodrugs, liposomes and other nanoparticles. TPGS has also have been used to function as an emulsifier, absorption enhancer and stabilizer, solubilizer, permeability enhancer as well as additive (Lu et al., 2014).

TPGS is an effective surfactant due to its amphiphilic property. Furthermore, TPGS has a large surface area which is vital for surface modification for conjugation of ligands. Moreover, TPGS can form micelles when dissolving in water at a concentration above its CMC value of 0.02 wt%. TPGS has been found to improve the half-life of drugs in plasma with the presence of PEG and vitamin E by enhancing cellular uptake of the drug molecules and hindering opsonisation. TPGS also holds advantages such as overcoming MDR, inhibiting membrane transporters, specifically P-gp efflux pump, increasing intestinal permeability and bioavailability of certain drugs. TPGS has also found usefulness in nanomedicine, given its capability to improve permeability and solubility of hydrophobic drugs and to afford controlled and sustained delivery of drugs (Kutty, 2015).

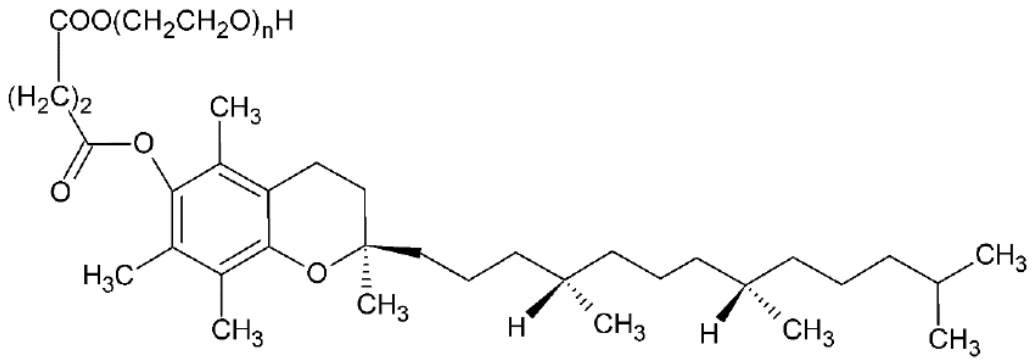


Figure 2.4: Molecular structure of Vitamin E TPGS (Kutty, 2015)

CHAPTER 3

METHODOLOGY

3.1 Introduction

In this research project, a micelle system of vitamin E TPGS for the delivery of docetaxel as a model anticancer drug for the treatment of TNBC whereas coumarin-6 as a fluorescence imaging agent for the diagnosis purpose of TNBC was developed. The micelles were characterized to obtain favourable particle size, drug loading, drug encapsulation efficiency and drug release profile. This chapter provides information on materials employed during micelle preparation and characterization. It also includes the supplier, brand and grade of chemicals used. Furthermore, detail descriptions of research methodology for micelles preparation and characterization are also presented in this chapter.

3.2 Materials

Docetaxel (anhydrous, 99.56% purity), Coumarin-6 and Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate, $C_{33}O_5H_{54}(CH_2CH_2O)_{23}$) were obtained from National University of Singapore (NUS). Chloroform (stabilised with 0.6-1.0% ethanol) was purchased from R&M Chemicals. Dichloromethane (DCM) and acetonitrile were all purchased from Fisher Scientific. Tween-80 was provided by Firma Chempur Company, Poland. Methanol was obtained from Fisher Scientific Corporation. Phosphate buffered saline (PBS, pH7.4) was prepared in the laboratory from sodium chloride (NaCl), potassium chloride (KCl), disodium phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4). All solvents used in this research study were high performance liquid chromatography (HPLC) grade. All chemicals were used without further

purification. Ultrapure water was prepared by a Milli-Q Plus System (Millipore Corporation, MA, USA). Amicon ultra-15 centrifugal filter units (molecular weight cut-off (MWCO): 10 kDa) were purchased from Merck Millipore (Billerica, MA).

3.3 Preparation of docetaxel loaded TPGS micelle (TPD), coumarin-6 loaded TPGS micelle (TPC6) and TPDC6 micelle

TPD and TPC6 micelles were prepared by solvent casting method. Briefly, docetaxel or coumarin-6 (2 mg) and 100 mg of TPGS were dissolved in 5 mL of chloroform solution. The organic solvent was evaporated using rotary vacuum evaporator (water bath temperature set to 20°C-30°C) until a thin film of drug-dispersed TPGS was formed. The thin film was then suspended in 10 mL of PBS buffer solution (pH 7.4) and incubated in an orbital water bath shaker at 37°C under constant agitation for 1.5 h and followed by 10 min of sonication. The resultant mixture was filtered through a 0.22-µm filter followed by centrifugal filter units with MWCO 10 kDa (Amicon ultra-15 ml) to separate the excess non-incorporated drug precipitate from the suspension. The resultant suspension was then freezed at -80°C for overnight followed by further dried in a freeze-dryer for overnight. The same procedure was used for the synthesis of TPDC6 micelle with replacement of 2 mg of docetaxel and 0.2 mg of coumarin-6 (10:1 weight ratio) and 100 mg of TPGS in 10 mL suspension. The theoretical weight (in mg) ratio of TPGS, docetaxel and coumarin-6 in the formed micelles was 97.84:1.96:0.2 for the 10 mL suspension (Kutty et al., 2015). Figure 3.1 represents the schematic illustration diagram of the formulation of TPDC6 micelle.

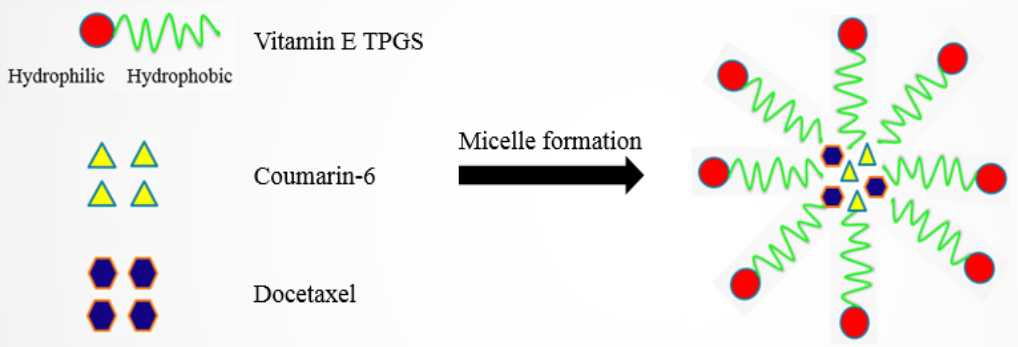


Figure 3.1: Schematic illustration of the formulation of TPDC6 micelle

(A) (B)

(C)

Figure 3.2: Photograph of equipment. (A) Rotary vacuum evaporator (B) Water bath shaker (C) Freeze dryer

3.4 Particle size and size distribution of micelles

The particle Size and size distribution of the TPD micelles, TPC6 micelles and TPDC6 micelles were measured by dynamic light scattering (DLS) using Zetasizer (Nano ZS, Malvern Instrument Ltd, Malvern, UK). The micelle samples were first prepared by diluting the nanoparticles suspension with ultrapure water to a count rate of 300-500 kilo counts per second (kcps) and then sonicated for 5 min before measurement to ensure that the micelles were well dispersed (Mi et al., 2012).

3.5 Drug loading and encapsulation efficiency of docetaxel

The amount of docetaxel encapsulated in TPDC6 micelles or TPD micelles was measured by high performance liquid chromatography (HPLC, Agilent LC1100, Agilent, Tokyo, Japan) in a reverse-phase column (Eclipse XDB-C18, 4.6 x 250 mm, 5µm). Briefly, 1 mL micelles were freeze-dried and dissolved in 1 mL of DCM to break polymer matrix. After evaporating of the DCM overnight, 0.5 mL of a mobile phase (50% acetonitrile in ultrapure water in volume ratio, 50:50 v/v) was added to dissolve the extracted drugs. After centrifugation at 13,000 rpm for 5 min, a supernatant of the suspension was collected and the solution was then filtered with a 0.45 µm PVDF syringe filter before being transferred into a HPLC vial for HPLC analysis. The flow rate of mobile phase was set at 1.0 mL/min and the column effluent was detected with an ultraviolet/visible (UV/VIS) detector at 230 nm, for the detection of docetaxel. The drug encapsulation efficiency was defined as the ratio between the amount of docetaxel encapsulated in the micelles and that added in the micelles preparation process. The drug loading was calculated as the weight of the drug encapsulated in the micelles divided by the total weight of the micelles (Muthu et al., 2015; Kutty, 2015).

$$Drug\ Loading\ = \frac{Weight\ of\ drug\ encapsulated}{Total\ weight\ of\ micelles} \times 100\%$$

Figure 3.3: Photograph of HPLC (Agilent LC1100)

3.6 Drug loading and encapsulation efficiency of coumarin-6

The drug loading and encapsulation efficiency were defined by measuring the amount of coumarin-6 encapsulated in TPDC6 micelles or TPC6 micelles through the following method. 1 mL of methanol was added into a designated amount of freeze-dried micelle sample and vortexed it until all the freeze-dried micelle was dissolved completely. A certain amount of micelles suspension was diluted three times with methanol. The concentration of coumarin-6 in the micelle was detected by the ultraviolet/visible (UV-VIS) spectrophotometer (Hitachi, U-1800) at 430 nm which is the excitation wavelength of coumarin-6. The total amount of encapsulated coumarin-6 was calculated (Zhao et al., 2014).

Figure 3.4: Photograph of UV-VIS Spectrophotometer (Hitachi U-1800)

3.7 *In vitro* drug release of docetaxel or coumarin-6 from micelles in buffer solution

The centrifugal ultrafiltration technique was used to study the *in vitro* drug release profile of docetaxel and coumarin-6 from micelles. 1 mL solutions of TPD micelles, TPC6 micelles and TPDC6 micelles were respectively added into the ultrafiltration membrane of centrifugal filter units with MWCO 10 kDa and immersed in 30 mL of PBS (0.1 M, neutral pH 7.4) containing 0.1% (v/v) Tween-80 in the filtrate collection cup of centrifugal filter unit. Since docetaxel and coumarin-6 are insoluble in the buffer solution,

0.1% v/v Tween-80 was added to simulate the sink condition. The entire system was kept at 37ºC with gentle and constant shaking. At designated time intervals, the incubation buffer in the filtrate collection cup was collected and replaced by a fresh incubation buffer. The collected incubation buffer, containing the released drug, was then freeze-dried and dissolved in DCM. The samples were filtered through 0.45 mm syringe filter before transferred into HPLC vial. The amount of docetaxel released was determined by the HPLC (Agilent LC1100, Agilent, Tokyo, Japan) method as described in the drug loading and encapsulation determination (section 3.5). The amount of coumarin-6 released was determined by the UV-VIS spectrophotometer (Hitachi, U-1800) as described in the protocols of drug loading and encapsulation determination (section 3.6). The drug release profile were calculated (Mi et al., 2012; Kutty et al., 2015).

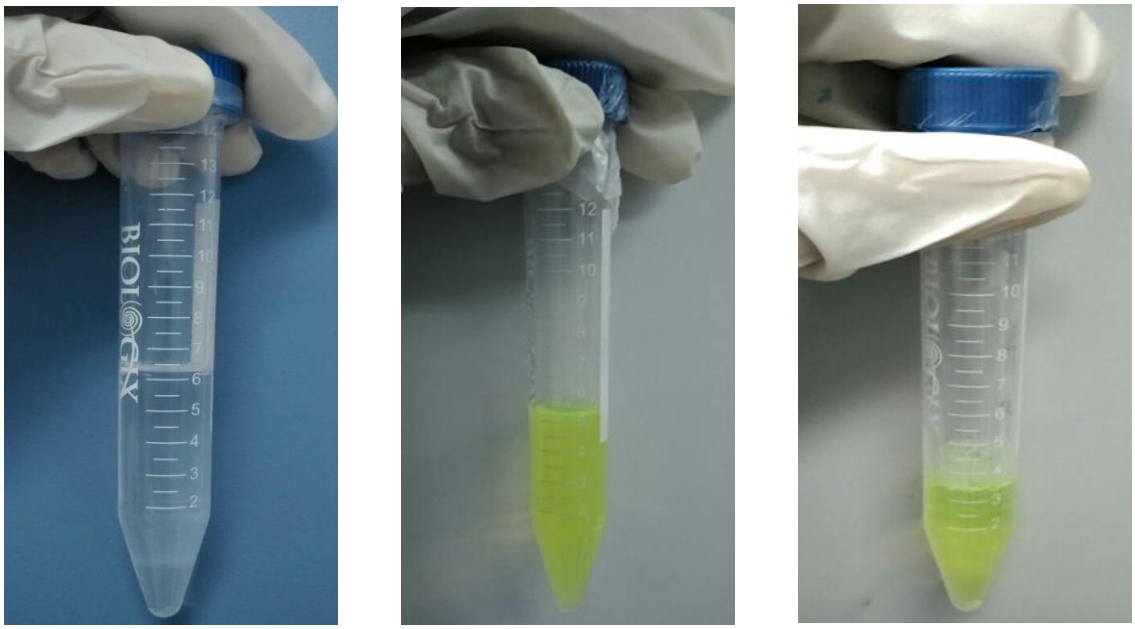
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of micelles

4.1.1 Micelle particle size analysis

The particle size of micelles measured by the dynamic light scattering (DLS) are shown in **Table 4.1** and **Figure 4.2**, from which it can found that the mean micellar size of the TPD micelles, TPC6 micelles and TPDC6 micelles were in the range from 20 nm to 80 nm. The mean sizes of TPD micelles, TPC6 micelles and TPDC6 micelles were 21.36 nm, 69.06 nm and 79.59 nm respectively, which were in a very favourable range of size for drug delivery in cancer treatment. It was revealed that micellar size typically in the range of 10 nm to 100 nm are appropriate for drug delivery in cancer diagnosis and therapy due to the reason of micelles in such size enhances EPR effect to solid tumour (Muthu et al., 2014; Kutty, 2015). The EPR phenomenon is based on the nanometer size range of micelles, leaky vasculature and impaired lymphatic drainage characteristics of the neoplastic tissues, which allowed the small size micelles (less than 100 nm) to participate in the extravasation through the fenestrations in tumour vessels and accumulate in the neoplastic tissues (Bazak et al., 2014; Steichen et al., 2012). According to Taurin et al. (2012), hydrodynamic diameter of more than 7 nm was able to escape from renal filtration and urinary excretion, leading to exhibit prolonged circulatory half-life in the blood and permit the accumulation of the nanoparticles within the neoplastic tissues. Therefore, TPD micelles, TPC6 micelles and TPDC6 micelles developed in this research could be an excellent delivery tools in cancer treatment due to the micelles size of less than 100 nm.

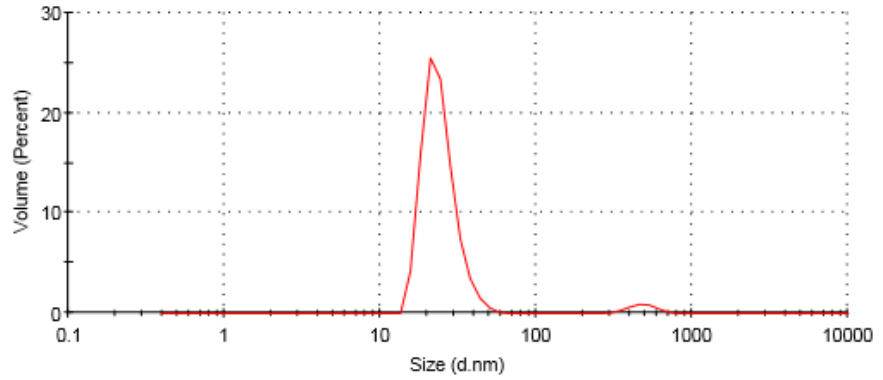


(a) (b) (c)

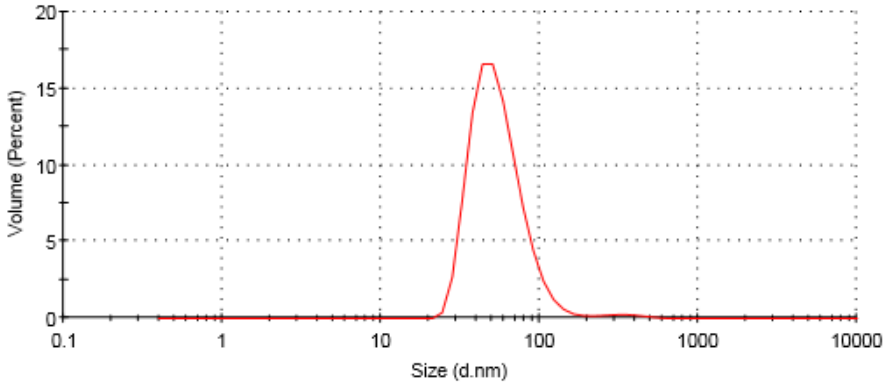
Figure 4.1: Photographs of nanoparticles. (a) TPD micelles (b) TPC6 micelles and (c) TPDC6 micelles

Table 4.1: Particle size of TPD micelles, TPC6 micelles and TPDC6 micelles

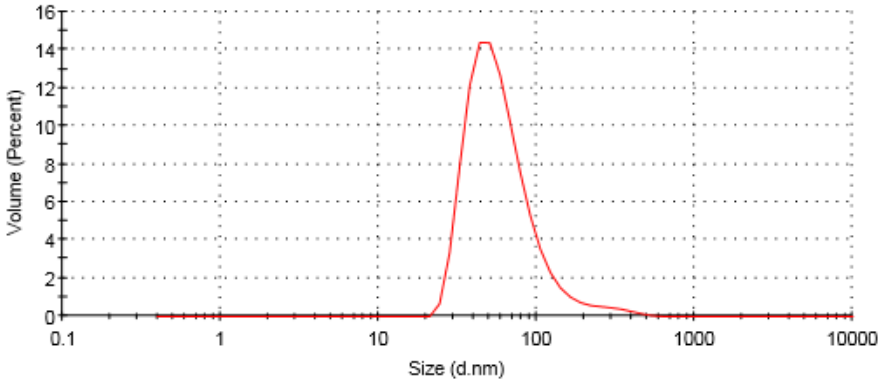
Micelles	**Mean size, nm**
TPD	21.36
TPC6	69.06
TPDC6	79.59



(A)



(B)



(C)

Figure 4.2: Particle size of the micelles. (A) TPD micelles (B) TPC6 micelles and (C) TPDC6 micelles

4.1.2 Drug loading and encapsulation efficiency

Drug load is determined as the weight of drug (µg) per mg of the drug-loaded micelles. The docetaxel loaded into the TPD and TPDC6 micelles was measured using HPLC whereas the coumarin-6 loaded into TPC6 and TPDC6 micelles was measured using UV-VIS spectrophotometer. The results are shown in **Table 4.2** and **Table 4.3**, the docetaxel load for TPD and TPDC6 micelles are 16.28 ± 1.33 µg/mg and 15.46 ± 0.56 µg/mg respectively while the drug loading of coumarin-6 for TPC6 and TPDC6 micelles are 10.60 ± 1.11 µg/mg and 9.83 ± 0.35 µg/mg respectively. The drug encapsulation efficiency of TPD micelles was 83.02 ± 6.81% whereas the dye encapsulation efficiency of TPC6 micelles was 54.05 ± 5.67%. The data are in close agreement with the research carried out by Muthu et al. (2012) for similar systems. Similarly, the drug encapsulation efficiency and dye encapsulation efficiency of TPDC6 micelles were 78.99 ± 2.88% and 36.20 ± 2.22% for docetaxel and coumarin-6 respectively.

The drug loading of coumarin-6 for both TPC6 micelles and TPDC6 micelles were lower compared to docetaxel loading, yet, it was high enough for in vivo tracking of nanoparticles and imaging purpose (Wu et al., 2014). The possibility for lower drug loading might be due to leakage of coumarin-6 and docetaxel during the incubation step. Furthermore, the relatively lower drug loading and encapsulation efficiency of coumarin-6 for TPDC6 micelles could be caused by the difference in hydrophobicity between docetaxel and coumarin-6. According to the study of Feng et al. (2014), coumarin-6 being more hydrophilic than docetaxel, however, Yagui et al. (2004) claimed that drug solubilization was more effective for the most hydrophobic drug than for more hydrophilic ones, hence, it caused lower drug loading and encapsulation for coumarin-6 in TPDC6 micelles. Moreover, the higher amount of docetaxel (2 mg) compared to coumarin-6 (0.2 mg) in the micelles preparation process might be one of the reason. The weight ratio of 10:1 and drug load of 15.46 µg/mg: 9.83 µg/mg for docetaxel and coumarin-6 respectively were adequate for the therapy purpose by docetaxel and imaging purpose by coumarin-6.

Table 4.2: Drug loading and encapsulation efficiency of docetaxel in TPD and TPDC6 micelles

Micelles	Drug load (µg/mg)	Encapsulation efficiency (%)
TPD	16.28 ± 1.33	83.02 ± 6.81
TPDC6	15.46 ± 0.56	78.99 ± 2.88

Table 4.3: Drug loading and encapsulation efficiency of coumarin-6 in TPC6 and TPDC6 micelles

Micelles	Drug load (µg/mg)	Encapsulation efficiency (%)
TPC6	10.60 ± 1.11	54.05 ± 5.67
TPDC6	9.83 ± 0.35	36.20 ± 2.22

4.2 *In vitro* drug release profile

In order to facilitate passive targeting to the tumour site, the drug should be released in a controlled manner and obtained sustained circulation to attain sustained therapeutic efficacy. *In vitro* release study was carried out in the medium of PBS (pH 7.4 with 0.1% v/v Tween-80) and frequent replacement of fresh buffer for simulating the sink condition. **Figure 4.3** and **Figure 4.4** show the *in vitro* drug release profiles of docetaxel and coumarin-6 respectively, in TPD, TPC6 and TPDC6 micelles for 240 hour. It can be seen from the figures that the drug release profile of the three micelle systems follow a biphasic pattern with an initial burst release followed by controlled release. The initial burst of docetaxel release was 55.25% and 21.62% in the first 50 hour, for the TPD and TPDC6 micelles respectively. Whereas, the initial burst of coumarin-6 was 16.30% and 10.70% in the first 48 hour, for the TPC6 and TPDC6 micelles respectively. The burst release could be due to the drug poorly encapsulated in the polymeric core and located under the periphery of the nanoparticles while the sustained release was attributed to the diffusion of the well entrapped drug in the core of nanoparticles (Tang et al., 2013).

After 10 days, the accumulative docetaxel drug release from TPD micelles achieved 64.99%, which was significantly higher than that from TPDC6 micelles with only 38.88% drug release. This might be due to the diffusivity of drug which depends on its solubility (Varma et al., 2004). As we discussed previously, coumarin-6 is more hydrophilic than docetaxel and this might be affected the diffusivity and drug release of docetaxel in TPDC6 micelles. Besides, Reza et al. (2003) revealed that the mechanisms of drug release was changed with the content in the polymer could be one of the reason for this phenomena. Whereas, the accumulative coumarin-6 drug release from TPC6 and TPDC6 micelles after 10 days were no significant difference observed, which were 21.63% and 16.86% respectively. The fluorescence probe coumarin-6 encapsulated within the nanoparticles was acting as tracers *in vivo* or *in vitro* due to their relatively low leak from nanoparticles and good sensitivity. The very low release of coumarin-6 from micelles indicated that coumarin-6 could be adequate fluorescence probes for micelles behaviour *in vivo*, and the fluorescence signals detected in the cells or tissue are well represented the distribution of the micelles instead of from release of coumarin-6 from the micelles into the medium (Kulkarni and Feng, 2011).

Overall, all the three micelle systems showed sustained and controlled release of drug, this implies that the drugs can be released slowly and kept for long period both *in vivo* and *in vitro* which causes repeated administrations is avoided leading to less drug accumulation and toxic effects in the body (Sharma et al., 2015; Zheng et al., 2009).

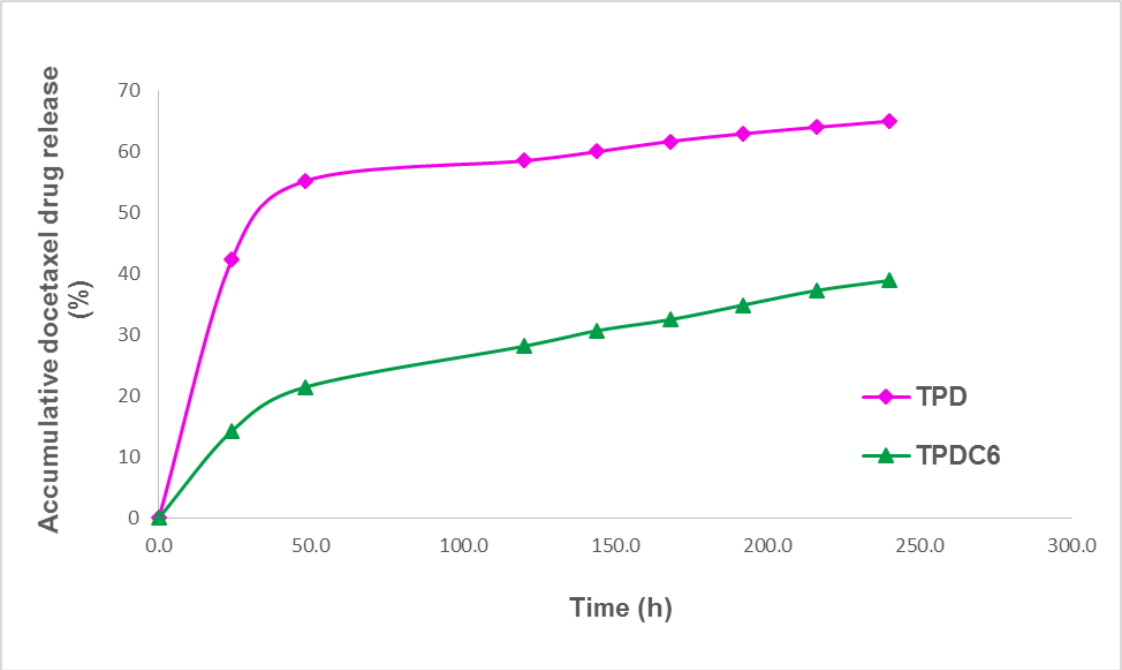


Figure 4.3: *In vitro* accumulative docetaxel drug release profile of TPD and TPDC6 micelles at pH=7.4. One-way ANOVA compared to TPD, *p < 0.05

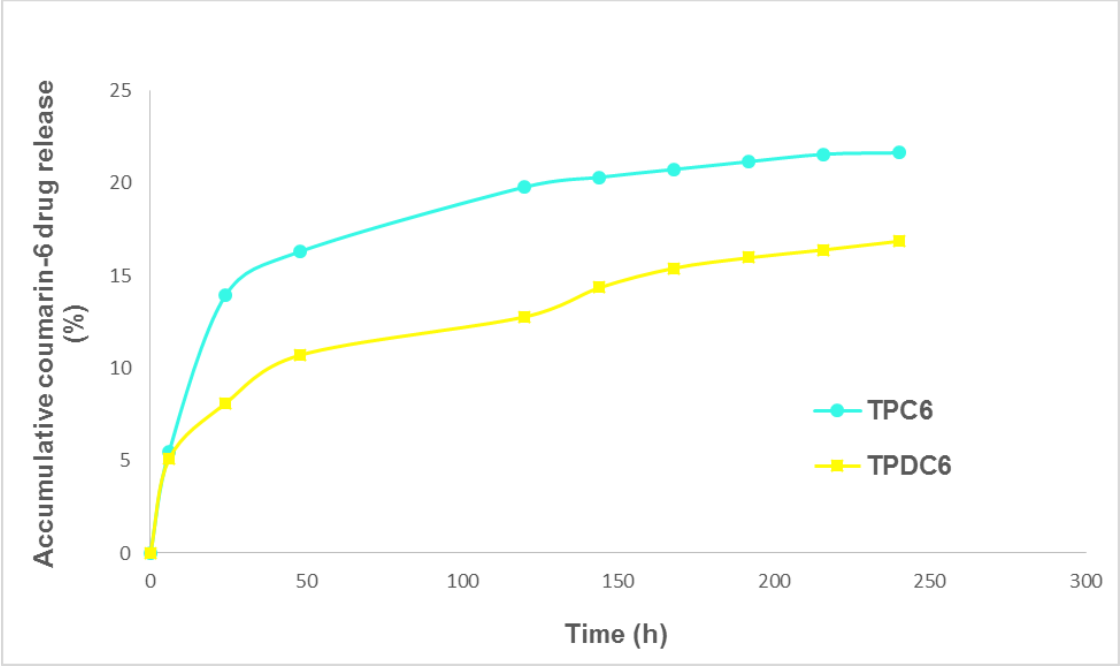


Figure 4.4: *In vitro* accumulative coumarin-6 drug release profile of TPC6 and TPDC6 micelles at pH=7.4. One-way ANOVA compared to TPC6, *p < 0.05

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In summary, a dual modality system was successfully developed using TPGS micelles for the sustainable and controlled delivery of docetaxel and coumarin-6 into the cancer cells. This research proved that the solvent casting method is considered suitable for the preparation of micelles with co-delivery of therapeutic and diagnostic agents. The DLS and particle size analyser showed that the TPDC6 micelles are in average nano size of 79.59 nm in diameter. The micelles showed drug loading up to 15.46 µg/mg (encapsulation efficiency of 78.99%) and 9.83 µg/mg (encapsulation efficiency of 36.20%) for docetaxel and coumarin-6 respectively. This results are adequate for the purposes of therapy and diagnosis on cancer cells by docetaxel and coumarin-6 respectively. *In vitro* drug release profiles demonstrated a desired sustained and controlled drug release pattern of the micelles which have advantages of less frequent drug administration, maximum utilization of drug and reduction in side effect of drug in the body.

5.2 Recommendation

There are few recommendations that should be considered for further study and investigation in order to improve the results from this research. From this study, the drug loading and encapsulation efficiency of the dual modality micelles (TPDC6 micelles) is not in the most excellent condition, hence, it is recommend to optimise the drug loading by varying the weight ratio of docetaxel, coumarin-6 and TPGS or change a different preparation method to develop the micelles in order to produce a smaller size micelles

with higher drug loading and encapsulation efficiency which would be very beneficial in drug delivery. Besides, the drug release profile of TPDC6 micelles in this study showed that the docetaxel drug release was slower and lower compared to TPD micelles. Therefore, further investigation on shape and surface morphology on the micelles could be conducted in order to understand the drug release mechanisms of TPDC6 micelles.

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APPENDIX

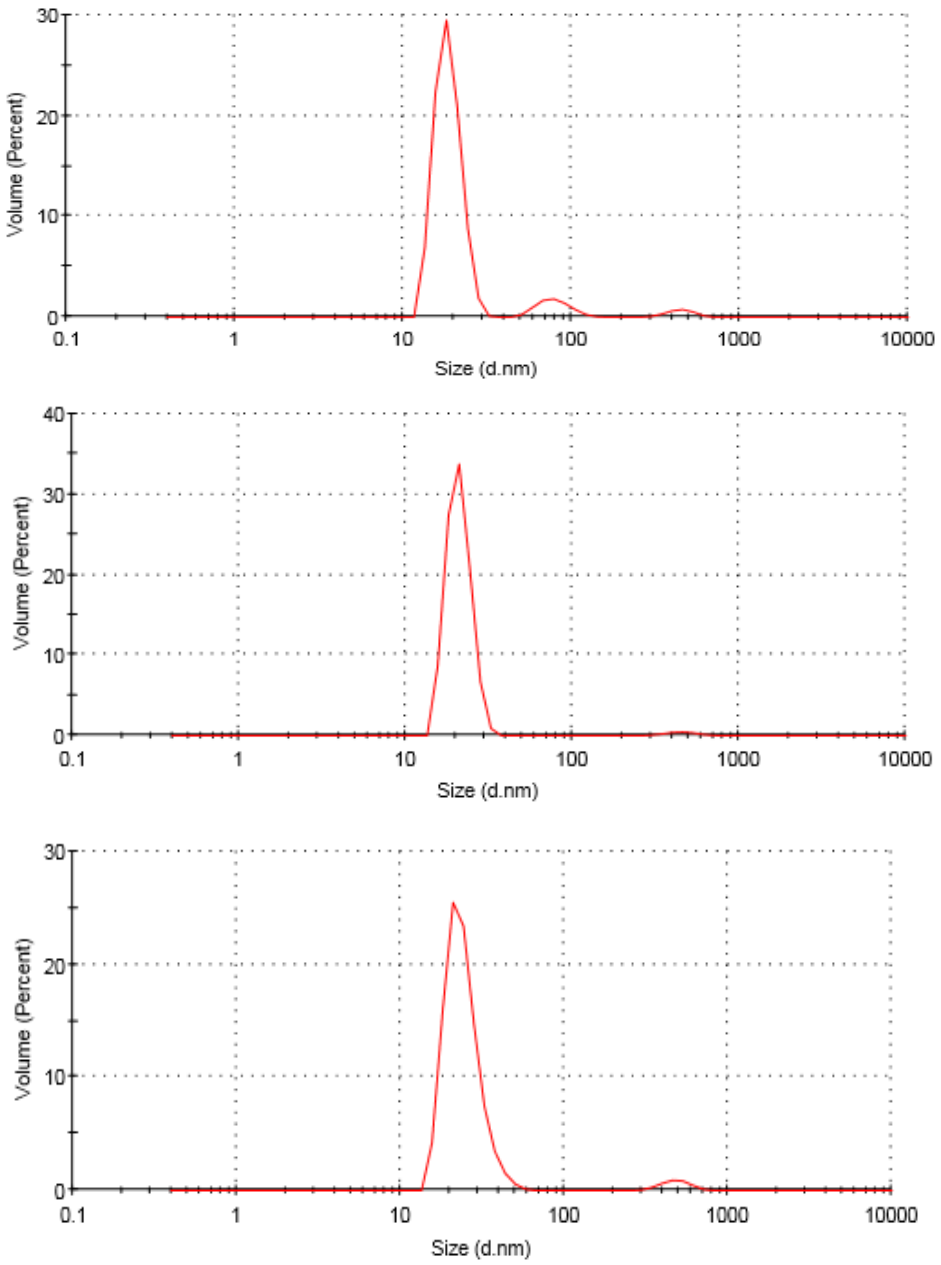

Figure A.1: Particle Size of TPD micelles

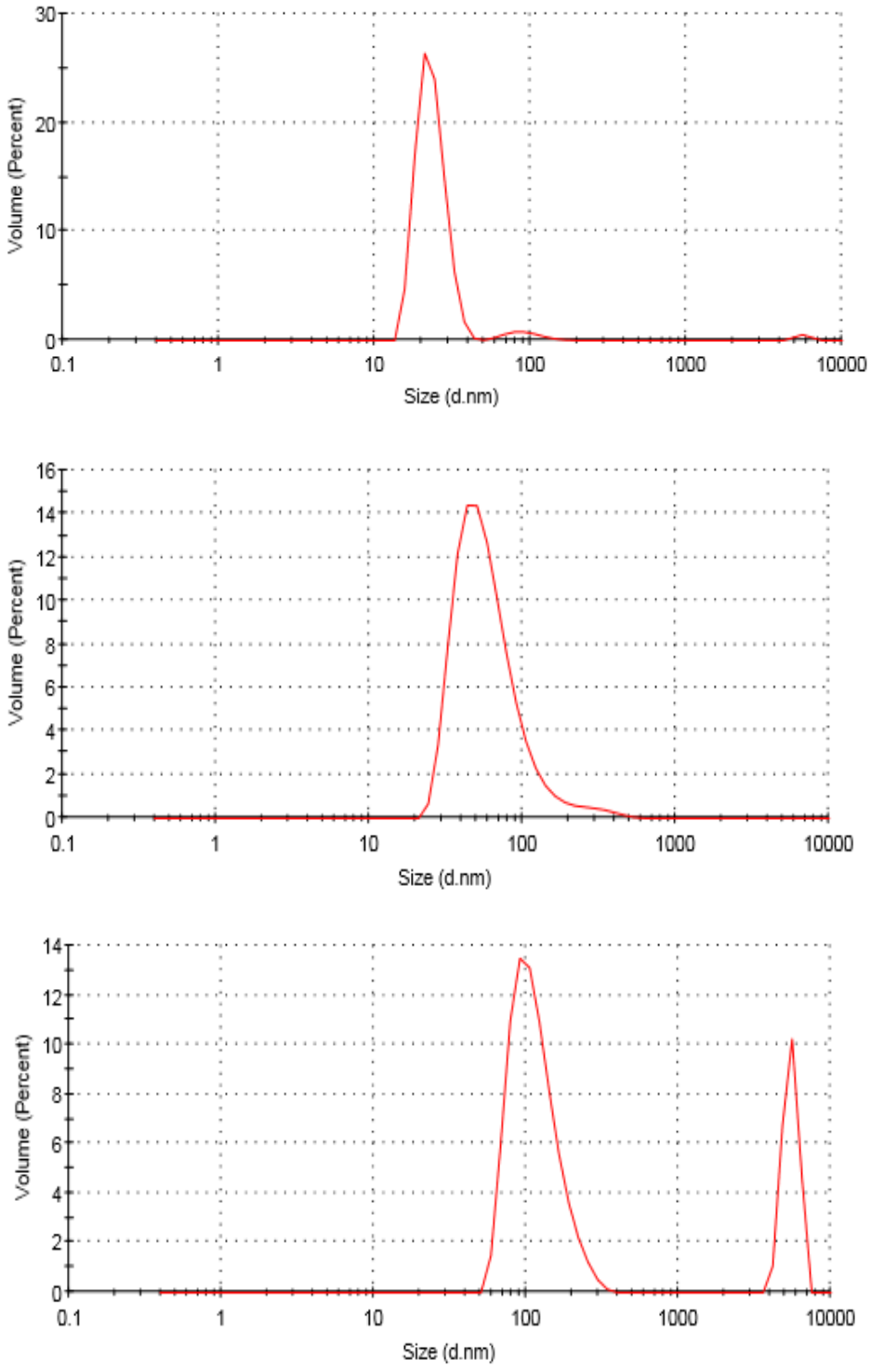

Figure A.2: Particle Size of TPC6 micelles

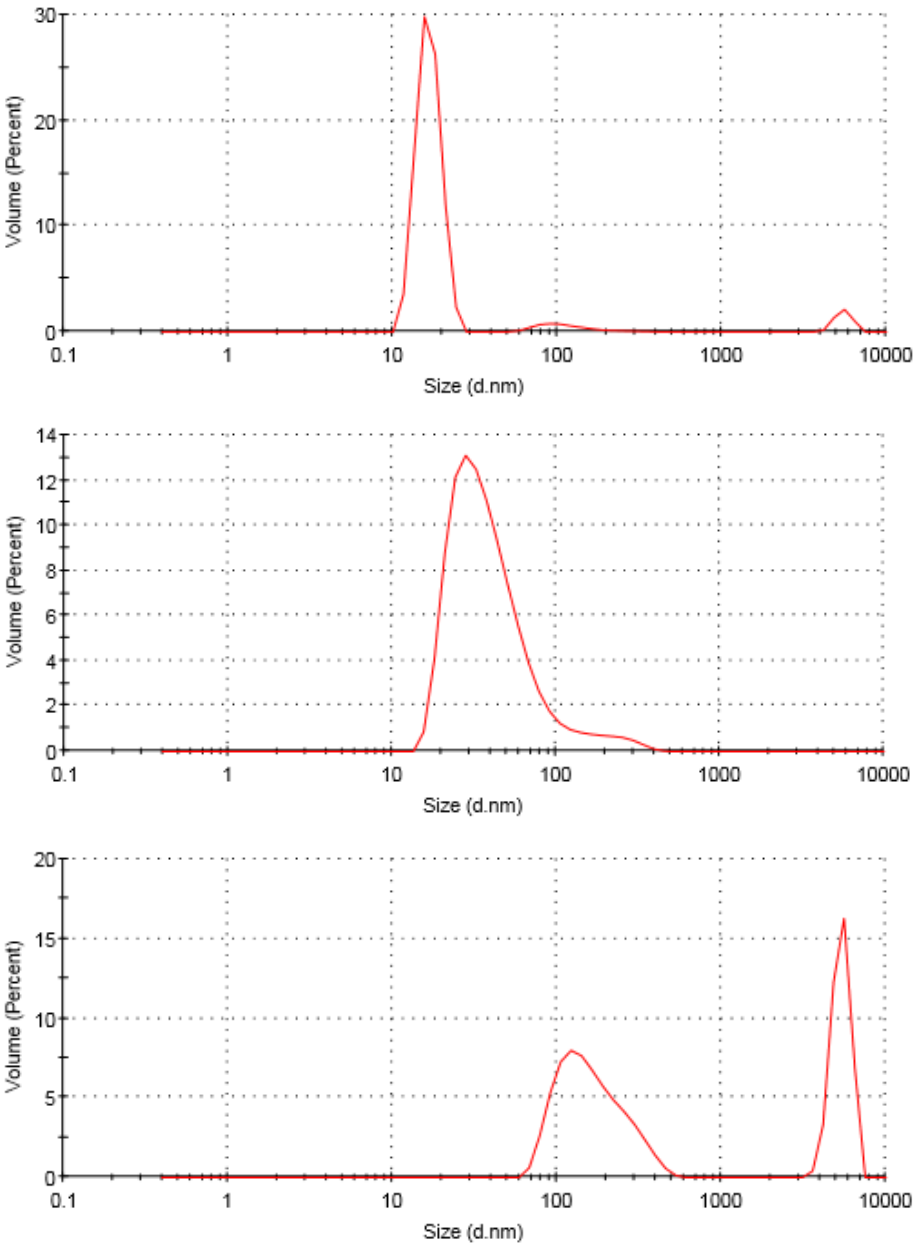

Figure A.3: Particle Size of TPDC6micelles

Table A.1: Triplicate result of particle size for TPD, TPC6 and TPDC6 micelles

Sample	Particle Size			Average
	1st	2nd	3rd	
TPD	18.70	21.07	24.30	21.36
TPC6	23.36	66.41	117.40	69.06
TPDC6	16.78	47.69	174.30	79.59

Table A.2: Drug loading and encapsulation efficiency of docetaxel for TPD micelles

Sample	Drug loading (ug/mg)	Encapsulation efficiency (%)
1	14.7541	75.25
2	17.2429	87.94
3	16.8354	85.86
Average	16.2775	83.02
STDEV	1.33	6.81

Table A.3: Drug loading and encapsulation efficiency of coumarin-6 for TPC6 micelles

Sample	Drug loading (ug/mg)	Encapsulation efficiency (%)
1	11.67	59.51
2	9.45	48.20
3	10.67	54.44
Average	10.60	54.05
STDEV	1.11	5.67

Table A.4: Drug loading and encapsulation efficiency of docetaxel for TPDC6 micelles

Sample	Drug loading (ug/mg)	Encapsulation efficiency (%)
2	14.8080	75.67
3	15.8083	80.78
4	15.7588	80.53
Average	15.4584	78.99
STDEV	0.56	2.88

Table A.5: Drug loading and encapsulation efficiency of coumarin-6 for TPDC6 micelles

Sample	Drug loading (ug/mg)	Encapsulation efficiency (%)
1	10.16	36.08
2	9.46	34.04
3	9.86	38.47
Average	9.83	36.20
STDEV	0.35	2.22

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	9	469.668	52.18533	429.9251		
Column 2	9	237.9183	26.43536	159.0995		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2983.775	1	2983.775	10.13124	0.005781	4.493998
Within Groups	4712.197	16	294.5123			
Total	7695.972	17				
Repeated Measure ANOVA - Within Subjects						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	
Between Groups	2983.775	1	2983.775	60.75388	5.264E-05	
Error	392.9	8	49.11249			
MS = SS/df						
F = $Ms_{between}/Ms_{error}$						
p Value = F.DIST.RT(F Value,$df_{between}$,df_{error})						

Figure A.4: ANOVA analysis for docetaxel drug release profile

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	160.9039	16.09039	57.2358
Column 2	10	115.6296	11.56296	31.42322

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	102.4882	1	102.4882	2.311963	0.145754	4.413873
Within Groups	797.9312	18	44.32951			
Total	900.4194	19				

Repeated Measure ANOVA - Within Subjects

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	102.4882	1	102.4882	36.71014	0.000188
Error	25.1264	9	2.791822		

MS = SS/df

$F = Ms_{between}/Ms_{error}$

p Value = F.DIST.RT(F Value,$df_{between}$,df_{error})

Figure A.5: ANOVA analysis for coumarin-6 drug release profile