PACLITAXEL LOADED NANOPARTICLES OF BIODEGRADABLE POLYMERS FOR CANCER CHEMOTHERAPY

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NATIONAL UNIVERSITY OF SINGAPORE

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Summary

The objective of this study was to develop a polymeric drug delivery system for an alternative formulation as well as for oral delivery of paclitaxel, which is used in our research as a prototype anticancer drug due to its excellent efficiency against a wide spectrum of cancers and its great commercial success as the best seller among antineoplastic agents, In our nanoparticle formulation, vitamin E TPGS (TPGS) is used as a necessary auxiliary in nanoparticle formulation as well as a "mask" for the nanoparticles to cross the GI barrier for oral chemotherapy. Paclitaxel-loaded, TPGS-emulsified poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles were prepared by a modified solvent extraction/evaporation single emulsion technique. Nanoparticle of various recipes were characterized by various state-of-the-art techniques such as laser light scattering for particle size and size distribution, scanning electron microscopy (SEM) for surface morphology, X-ray photoelectron spectroscopy (XPS) for surface chemistry, and high performance liquid chromatography (HPLC) for *in vitro* drug release kinetics. Caco-2 cells were employed as an established *in vitro* model of the GI barrier. Human colon adenocarcinoma cells (HT-29 cells) were used to evaluate the cytotoxicity of the drug formulated in the nanoparticles, which was measured in a close comparison with its current clinical dosage form Taxol®. *In vivo* pharmacokinetics was also determined and compared with Taxol®.

The formulated nanoparticles were found in quite uniform size of ~240 nm diameter. The *in vitro* drug release profile exhibited a biphasic pattern with an initial burst followed by a sustained release. Uptake of fluorescent nanoparticles by Caco-2 cells was evidenced by confocal microscopy, which was found strongly dependent on the size and surface coating of the nanoparticles. *In vitro* HT-29 cell viability experiment

demonstrated that the drug formulated in the nanoparticles theoretically could be 46.18, 41.64, 19.65, 10.47 times more effective than that in Cremophor EL formulation (Taxol®) after 24, 48, 72, 96 hours treatment, respectively at 0.25 μg/ml paclitaxel concentration. *In vivo* PK measurement also showed advantages of the nanoparticle formulation versus Taxol®. Vitamin E TPGS emulsified PLGA nanoparticle formulation of paclitaxel has advantages versus Taxol and may provide an ideal solution for the problems caused by Cremophor EL. The technology may also apply to other anticancer drugs.

CHAPTER 1

INTRODUCTION

1.1 Introduction

In order to improve the patient compliance and drug performance, pharmaceutical formulation researchers have been driven to design controlled release devices to deliver small molecule drugs, peptides and proteins, genes, and vaccines. Since a new formulation may extend the patent expire time of the specific drugs, the pharmaceutical companies are cooperating with research institutes to make the controlled release formulation available on the market.

The controlled drug delivery systems draw increased attention due to its enhanced efficacy of existing potent drugs at lesser expenses and fewer dosing schedule. Controlled delivery system maintains the drug level in the blood between the maximum and minimum therapeutic levels at a minimum dosage for an extended period of time (Karsa, 1996; Dunn, 1991). Conventional delivery system provides fluctuated drug level in the blood, either exceeding the maximum or falling below the minimum therapeutic level, resulting in toxic side effects or inefficacy.

Most anticancer drugs have limitations in clinical administration due to their poor solubility and other unfavorable properties. Paclitaxel is chosen as model drug for this study since it has shown promising antineoplastic activity for a wide spectrum of cancers, particularly against drug-refractory ovarian (Runowicz et al., 1993) and breast cancer

(Holmes et al., 1991). Currently, only available dosage form is Taxol® for intravenous (i.v.) injection, which is cumbersome for the patients and limits the use of frequent dosing schedule for a prolonged systemic exposure to the drug. Due to its high hydrophobicity, adjuvant as Cremophor EL (CrEL) has to be used, which is responsible for serious side effects (Lehoczky, 2001). Thus, the development of successful paclitaxel delivery system devoid of CrEL is essential for a better clinical administration with less side effects.

Although phase I study showed that co-administration of paclitaxel with P-gp inhibitors such as cyclosporine A increased oral bioavailability and it may be a medical solution for oral chemotherapy (Malingre et al., 2000b; Britten et al., 2000), cyclosporine A itself is an immunosuppressive agent and may cause severe nephropathies. Other types of P-gp inhibitors are also costly and need premedications to reduce side effects (Asperen et al., 1997; Malingrè et al., 2001a). Thus, this approach is not successful at the moment.

Biodegradable and bioadhesive nanoparticulate carriers could be an ideal solution for intravenous or oral delivery of paclitaxel as well as of other anticancer drugs. Biodegradable and biocompatible polymer prevents adverse effects and accumulation of polymer in the body over long-term application. Bioadhesive nanoparticulate based drug delivery system has been shown to increase oral bioavailability of drugs due to the increased residence time of the nanoparticulates within the gastrointestinal (GI) tract and increased contact time with the intestinal epithelium and hence increased uptake. Moreover, appropriate coating of nanoparticles may provide engineering make-ups to escape from the recognition of P-gp and improve interaction with the endothelial cells.

1.2 Objective of study

The aim of this study is to develop a new product of biodegradable polymeric nanoparticles for clinical administration of paclitaxel with less side effects, and with further modification, to promote oral chemotherapy. This system may also be applied to other anti-cancer drugs. Several additives including natural additives are applied not only to improve the adhesion and interaction of the nanoparticles with intestinal cells but also to act as emulsifier and solubilizer in the preparation process. The effects of various emulsifiers are investigated for the fabrication of biodegradable nanoparticles in an effort to achieve desirable properties for effective sustained release of drug with maximum drug efficacy. The physicochemical properties of nanoparticles are characterized by various state-of-the-art techniques. The in vitro release of the drug-loaded particles is also examined to study in greater detail of the release kinetics of the drug used and is modified by optimizing the preparation parameters. Evaluation of the effectiveness of the formulated nanoparticles delivery system in the *in vitro* cell line experiments and *in vivo* animal tests are performed to closely study the interaction between cells and polymeric particles, the particle uptake and to evaluate the efficacy and feasibility of the formulated delivery system.

1.3 Significance of Study

New dosage forms under development in this study may reduce side effects caused by both the anticancer drug and the adjuvant to provide possible improved efficacy. Intravenous administration of paclitaxel using biodegradable nanoparticulate system will 1) eliminate possible irritant reactions, 2) reduce systemic side effects, and 3) provide

sustained release. Potential administration of biodegradable nanoparticulate system via oral route will: 1) obviate the difficulties of the i.v. access, 2) improve quality of life of patients, 3) reduce side effects, 4) increase efficacy at lower dosage for longer time saving scarce drug, and 5) offer convenience to the patients eliminating the needs for hospitalization, physicians and nursing assistance and infusion equipment. This system, applying novel functional surfactants, has a potential to overcome the multi-drug resistance (MDR) of paclitaxel, which has been another serious problem in the clinical administration of paclitaxel. This novel oral formulation of paclitaxel may be developed into a completely new form of cancer chemotherapy.

1.4. Thesis Organization

This thesis comprises of 6 chapters. Chapter 1 presents a brief introduction, objective and significance of study. Chapter 2 provides a background understanding of cancer and its treatment, novel anticancer drug paclitaxel and its chemotherapy, and how biodegradable polymeric nanoparticles can be employed as drug delivery systems. Chapter 3 discusses the formulation and characterization of PLGA nanoparticles for oral paclitaxel administration. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs were investigated and detailed in Chapter 4. PLGA nanoparticles formulation for paclitaxel delivery was evaluated and discussed in Chapter 5 with extensive in vitro and in vivo studies for its drug release profile, cellular uptake, cytotoxicity and pharmacokinetics. Chapter 6 summarizes the findings of this study and gives recommendations for future work. List of publications stem out from this study and achievements are given in Appendix A and Appendix B, respectively.

CHAPTER 2

LITERATURE REVIEW

2.1. Cancer and Cancer Treatment

2.1.1. What is cancer?

Cancer is a group of diseases characterized by the uncontrolled growth of abnormal cells that disrupt body tissue, metabolism, etc. and tend to spread locally and to distant parts of the body. Humans are made up of cells and the normal cells divide and grow at a controlled rate. Cancer initiates as a change in the gene of a single normal cell in any part of the body. Once this change takes place, the set of instructions in the gene is changed and the cell becomes abnormal which no longer acts like it normally does. Cancer is actually due to the accumulation of many such errors. Life-threatening cancer develops gradually as a result of a complex mix of factors such as complex interactions of viruses, a person's genetic make-up, their immune response and their exposure to other risk factors which may favor the cancer. The notion of cancer as a serious, life-threatening disease must be very ancient; and probably for a long time different cultures have speculated that both external and internal factors play a role in the cause of cancer.

Cancer is the second leading cause of death in the United States closely following the heart diseases. The statistics report from National Cancer Institute (2002) stated that men have a little less than 1 in 2 lifetime risk of developing cancer while women have a little more than 1 in 3, in the US. That implies 30% of all Americans will develop some kind of cancer in their lifetimes. Cancers of the prostate and breast will be the

most frequently diagnosed cancers in men and women, respectively, followed by lung and colorectal cancers both in men and in women (cancer statistic, 2004). In Sweden, the incidence is approximately the same in the US, more than one fifth of all deaths is due to cancer. In Singapore, cancer is the leading cause of death (27% of total deaths) which means about one in four deaths is from cancer (Hock, 2002). Breast cancer is the leading cancer in Singaporean women, accounting for 20% of the cases (Chia, 1996).

The occurrence of cancer leads to pain, suffering and psychological harm to the patients and their families, but it is also an economic issue. In the United States, approximately 16 million new cancer cases have been diagnosed since 1990 and the overall costs for cancer in 2001 were estimated at $156.7 billion. It is estimated that 1.37 million new cases of cancer will be diagnosed in 2004 and one in every four deaths will be caused by cancer (American Cancer Society, 2002). More than 10 million people are diagnosed with cancer every year. It is estimated that there will be 15 million new cases every year by 2020. Cancer causes 6 million deaths every year or 12% of deaths worldwide (http://www.who.int/cancer/en/).

Although our current understanding of what causes cancer is not complete, we now know enough to prevent at least one-third of all cancers. Cancer is largely preventable: by stopping smoking, providing healthy food and avoiding the exposure to carcinogens. Information is also available that would permit the early detection and effective treatment of a further one-third of cases. The chance of cure increases substantially if cancer is detected early. Cancer control is a public health approach aimed at reducing causes and consequences of cancer by translating our knowledge into practice.

2.1.2. How to treat cancer?

Cancer treatment is a multidisciplinary therapy consisting of surgery, radiotherapy, chemotherapy and immunotherapy (Jönsson and Karlsson, 1990). The treatment sometimes has a curative intent, sometimes a palliative intent.

One typical therapeutic approach to solid tumor is surgical removal followed by irradiation and/or systemic chemotherapy to kill malignant cells which may have survived the surgery, and prevent metastasis and re-growth of the tumor. Surgery may leave unavoidable residual cancer cells and have undesirable side effect of changing the growth rate of the remaining cancer cells by triggering a faster metastatic process. Thus, multimodal therapy that comprises radiotherapy, chemotherapy, immunotherapy, and other forms of treatments follows the surgery to provide a better chance to kill the metastatic cancer cells or at least to keep them in the remission state.

The choice of treatment depends on the type and location of the cancer, whether the disease has spread, the patient's age and general health, and other factors (NCI, 2000). In many cases, especially for early stage cancer, undetectable cancer, metastatic cancer, or non-solid-tumor cancer (e.g. leukemia), chemotherapy has been proved to be necessary and effective treatment. Over the last decade, the situations have imposed the clinicians and researchers to aware the increasing demand of patients' quality of life in cancer treatments (Gotay et al., 1992). The ultimate goal of treatment is to increase life span and/or improve the quality of life for the patients.

Although great effort has been made in cancer research, no substantial progress can be observed in the past fifty years in fighting against cancer. The death rate in the USA was 193.9 per 100,000 in 1950 and remained as high as 194.0 per 100,000 in 2001

(Cancer Statistic, 2004). It is clear that the progress in cancer treatment has been slow and inefficient. Significant increments in cure rate are unlikely to be achieved unless more profound knowledge of cancer pathophysiology can be pursued, new anti-cancer agents discovered, novel biomedical technologies developed. It is a multidisciplinary challenge needing more and closer collaboration between clinicians, medical and biomedical scientists and biomedical engineers to eventually find a satisfactory solution.

2.1.3. Chemotherapy and anti-cancer drugs

Chemotherapy for cancer is treatment of cancer using therapeutical agents that have direct tumor-killing properties. Drugs that are specifically designated as part of hormone therapy and immunotherapy are sometimes included. Chemotherapy is most effective against cancers that divide rapidly and have a good blood supply. Aims of chemotherapy treatments are to cure; to maintain long term remission (free of disease); to increase the effectiveness of surgery or radiotherapy; to help control pain or other symptoms.

Drugs that are effective in treating cancer interfere with the activity of cancer cells, either by going in directly to sabotage a specific phase of cell development or by sending confusing messages that cause the cells to destroy themselves. Not all drugs are effective against all cancers, and the different groups of drugs act in different way.

Chemotherapy drug doses and schedules are developed so that the drugs enter the body, kill the rapidly dividing cancer cells, and are expelled before they can damage most healthy cells, which divide more slowly. But the normal cells that make up the

mucous lining of the intestinal tract, the hair producing cells, and the bone-marrow cells are also rapidly-dividing cells, hence these, too, are affected by the chemicals, causing the three most common side effects: nausea and vomiting, hair loss, and bone-marrow depression. Different drugs may cause different side effects and/or people may react differently to the same drug – some people have no side effects; some people have all of them; and most people fall somewhere in between.

2.2. Paclitaxel and chemotherapy

Chemotherapy is an effective treatment for cancer and other serious diseases such as cardiovascular restenosis and AIDS. Among the available drugs for chemotherapy, paclitaxel (Taxol®) is one of the best anti-cancer drugs and also reported to possess radio-sensitizer properties.

2.2.1. Paclitaxel: promising anti-cancer drug

Paclitaxel (5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one 4,10-diacetate-2-benzoate-13-ester with (2R, 3S)-N-benzoyl-3-phenylisoseine), is a white to off-white crystalline powder with empirical formula of $C_{47}H_{51}NO_{14}$ and a molecular weight of 853.9. It is highly lipophilic, insoluble in water, and melts at around 216-217°C. It is a complex, oxygen-rich diterpenoid (Rowinsky and Donehower, 1995; Rowinsky et al., 1992) and its chemical structure has been elucidated by chemists as in Fig. 2.1. It consists of some benzene rings and other hydrophobic structures, which lead to its high water insolubility of paclitaxel.

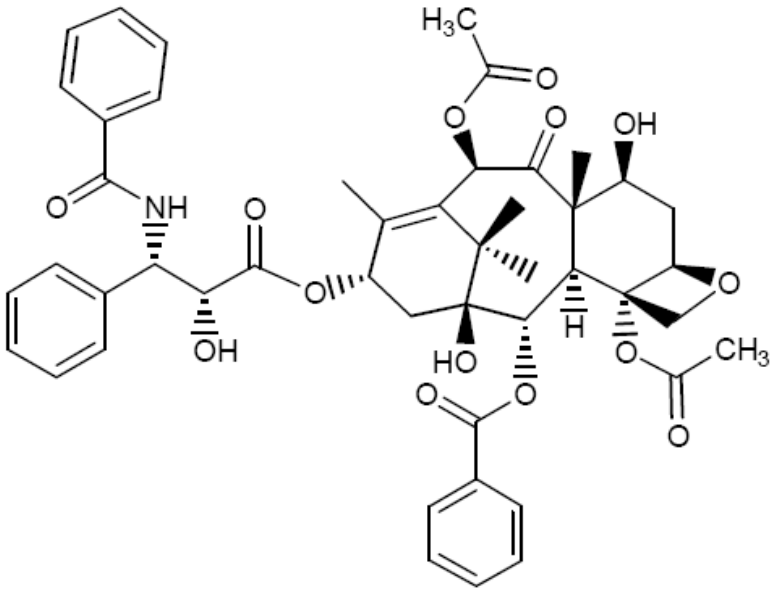


Figure 2. 1. Chemical structure of paclitaxel.

Paclitaxel was originally isolated from the bark of the Pacific yew tree *(Taxus brevifoli*a). Its anti-tumor activity was first detected in 1967 when the US National Cancer Institute (NCI) was carrying out tests to screen for the possible presence of cytotoxic agents from natural products. The growing demand of paclitaxel, limitations of resources and environmental concerns led to the production of a semi-synthetic form of paclitaxel derived from the needles and twigs of the Himalayan yew tree *(Taxus bacatt*a), which is a renewable resource. The FDA (Food and Drug Administration) approved the semi-synthetic form of paclitaxel in the spring of 1995.

Phase I and II clinical studies have demonstrated the significant activity of paclitaxel against a variety of solid tumors (Rowinsky et al., 1990; Holmes et al., 1991; Runowicz et al., 1993; Spencer and Faulds, 1994; Rowinsky and Donehower, 1995) including breast cancer, advanced ovarian carcinoma, lung cancer, head and neck carcinoma, colon cancer, multiple myeloma, melanoma, AIDS-related Kaposi's

sarcoma and acute leukemias. In 1992, it was approved by the US Food and Drug Administration (FDA) for ovarian cancer, later in 1994, for advanced breast cancer and then for early stage breast cancer in October 1999.

Reports have shown brain tumors are sensitive to the paclitaxel *in vitro* (Cahan et al., 1994) and it can also act as a radio-sensitizer for glioma cells *in vitro* (Tishler et al., 1992). Recent studies using paclitaxel as the radio-sensitizer have shown that exposure to very low concentrations (10 – 100nM) elicits potent clinical anti-neoplastic activity against a variety of advanced solid human tumors (Bissett and Kaye, 1993).

Paclitaxel has a low therapeutic index, and the therapeutic response is always associated with toxic side-effects (Terwogt et al., 1997). The major side effects include: depression of bone marrow, reduction of blood cell production, reversible hair loss, gastrointestinal problems, nerve damage, and so on (Kohler and Goldspiel, 1994). However, the excellent therapeutic efficacy outweighs the risks associated with paclitaxel.

2.2.2. Anticancer mechanism of paclitaxel

Paclitaxel, a prototype for a new class of anticancer drugs, has a unique way of preventing the growth and separation of cancer cells: it promotes microtubules assembly (Park, 1997). Microtubules, made up of numerous tubulins, are a necessary and essential component in cells which carry out many important cellular functions, such as nutrition ingestion, sensory transduction, cell movement, cell shape control and spindle formation during cell division. In normal cell growth, microtubules are formed when a cell starts dividing. Once the cell stops dividing, the microtubules are

broken down or destroyed. Paclitaxel stops the microtubules from breaking down, but it promotes and stabilizes microtubule assembly by non-covalent interaction with tubulin, thereby blocking cell replication in the late G2 mitotic phase of the cell cycle (Kumar, 1981; Manfredi and Horwitz, 1984; Horwitz, 1994). Cancer cells become so clogged with microtubules that they cannot grow and divide and thus lead to cell death (NCI, 2001; Guchelaar et al., 1994; Lopes et al., 1993; Brown et al., 1991; Rowinsky et al., 1990; Donehower et al., 1978). This mechanism of inhibition is unique from that of other mitotic inhibitors such as vinca alkaloids, which inhibit microtubule assembly (Ettinger et al., 1995).

2.2.3. Clinical administrations of paclitaxel

Since paclitaxel is highly hydrophobic (water solubility ≤ 0.5mg/L) (Liggins et al., 1997; Straubinger and Sharma, 1995) and not absorbed from the GI tract due to multi-drug resistant (MDR) transporter, it is mainly given by intravenous (i.v.) administration. Due to its poor solubility in conventional aqueous vehicles, the only available dosage form of paclitaxel, Taxol®, is formulated in a mixture of Cremophor EL (polyoxyethylated 35 castor oil) and dehydrated alcohol (1:1, v/v) as a concentrated solution containing 6 mg paclitaxel per ml. Cremophor is an excipient in many drug formulations used to overcome poor water solubility. However, it is associated with leaching of plastic from standard IV tubing, hypersensitivity reactions, complement activation, axonal swelling, and demyelination and may interact with paclitaxel to cause myelosuppression; it may also contribute to reduced cell penetration by encapsulating the drug.

2.2.3.1. Intravenous (i.v.) administration of paclitaxel

Taxol® must be further diluted 5 to 20-folds with 0.9% sodium chloride injection or other aqueous i.v. solutions before i.v. administration (Goldspiel, 1994). The drawback of this formulation is its short term stability upon dilution; it was found physically stable for only 3 h when diluted to drug concentrations ≤ 0.6mg/ml (Boyle and Goldspiel, 1998). Taxol® is generally given at a dose of 135 or 175 mg/m^2 as a 3 or 24 h infusion, every 3 weeks (Kramer and Heuser, 1995; Seidman et al., 1995). Premedication with corticosteroids (e.g. dexamethasone), diphenhydramine and antihistamine (both H1 and H2-receptor antagonist, e.g. cimetidine, ranitidine) is used to increase safety and reduce intensity and the incidence of serious hypersensitivity reactions associated with Cremophor-based paclitaxel administration (Weiss et al., 1990; Goldspiel, 1994; Lam et al., 1997).

It is now well established that the CrEL plays a major role in the hypersensitivity reactions (Miller and Sledge, 1999; Schiller, 1998; Spencer and Faulds, 1994; Jordan et al., 1993; Horwitz et al., 1986; Schiff et al., 1979). The less known side effects of CrEL are neurotoxicity, nephrotoxicity and cardiotoxicity (Zuylen, et al., 2001; Lehoczky et al., 2001). Although some studies suggested that CrEL and Tween 80 may enhance taxane cytotoxicity (Nygren et al., 1995) and might have a cytotoxic effect of their own (Fjällskog et al., 1993), this could not be confirmed by others (Terzis et al., 1997).

It was also reported that CrEL has influence on the functions of endothelial and vascular muscle, leading to vasodilation, laboured breathing, lethargy, hypotension and toxicity to myocardium. Formulation of paclitaxel, with Cremophor and ethanol, precipitates upon dilution with infusion fluid and also during storage for extended

time periods (Lewis, 1990). Hence, an in-line filter is recommended for the intravenous line and it is suggested that drug is administered promptly after dilution.

Paclitaxel formulation in ethanol:CrEL mixture also shows an incompatibility with the components of the infusion sets. It was reported (Venkataraman et al., 1986; Pfeifer and Hale, 1993; Rowinsky et al., 1993; Fjallskog et al., 1993; Dorr, 1994; Goldspiel, 1994; Allwood and Martin, 1996; Song et al., 1996; Xu et al., 1998) that both ethanol and Cremophor leach diethylhexylpthalate (DHEP) from the polyvinylchloride (PVC) containers and intravenous infusion line. The manufacturers of paclitaxel thus recommended the use of glass, polypropylene or polyolefin containers for its storage. These recommendations however, pose a number of practical problems since the availability of these types of containers is severely limited and as such the medical staff may be unfamiliar in its handling.

Hence, it becomes necessary to come up with alternative dosage forms that are capable of overcoming the problems of CrEl.

2.2.3.2. Oral administration of paclitaxel

Oral route is the easiest and the most convenient way of drug administration, especially when repeated or routine dosing is required (Florence and Jani, 1993). Oral administration of paclitaxel is to be preferred as it may circumvent the use of CrEL and it offers convenience to the patients and improves the quality of life of the patients. It facilitates more chronic treatment regimens and promotes long exposure to the drug, which may have pharmacodynamic advantages and can thus improve the efficacy.

Unfortunately, oral paclitaxel is poorly bioavailable due to rapid elimination from the body by the first pass effect of cytochrome P450 (CYP) dependent metabolic process; and its high affinity for the plasma membrane multidrug transporter P-glycoprotein (P-gp) which is abundantly present in the GI tract (Dintaman and Silverman, 1999; Panchagnula, 1998; Seidman et al., 1995; Lopes et al., 1993; Rowinsky et al., 1990; Link et al., 1995; Donehower et al., 1987).

2.2.4. Limitations of clinical paclitaxel formulations

The main limitation for clinical application of paclitaxel is its low solubility in water and most of the pharmaceutical solvents. The reported water solubility of paclitaxel varies from ~7 μM (~6 mg/L) to 35 μM (~30 mg/L), depending on the determination method employed (Tarr and Yalkowsky, 1987; Swindell et al., 1991). Its solubility cannot be improved by the manipulation in pH because its molecules lack functional groups that are ionizable within the pharmaceutically useful pH range. The common approaches to enhance solubility such as the addition of charged complexing agents, the production of alternate salt forms, etc. are not applicable in the case of paclitaxel (Straubinger, 1995).

The major obstacle in formulating successful oral paclitaxel dosage form is P-gp in the mucosa of the small and large intestine which limits the oral uptake of paclitaxel and mediates direct excretion of the drug in the intestinal lumen (Adams et al., 1993). This P-gp limitation was confirmed by some studies. The systemic exposure of oral paclitaxel was found 6-folds higher in mdr1a knockout mice model (lacking functional P-gp in the gut) than in wild-type mice (Asperen et al., 1996). High systemic availability could be achieved in wild-type mice when paclitaxel was

administered per-oral in combination with SDZ PSC 833 or with CsA, both are efficacious P-gp inhibitors (Asperen et al., 1997). However, these P-gp inhibitors may also suppress immune system and propose side effects over long-term application. Moreover, the cost of these inhibitors is another hindrance for successful development of oral dosage form of paclitaxel.

Another setback is the requirement of relatively large doses of paclitaxel for a complete block of cell proliferation. Paclitaxel concentration required to completely inhibit cell growth is in excess of 10, 000 folds of that required to inhibit tumor cell growth by 50% (IC_{50}) which is in nanomolar range (Spencer and Fraulds, 1994).

The most serious clinical problem of the current paclitaxel formulation is hypersensitivity reactions due to its toxic adjuvant, CrEl and ethanol mixture. These problems were observed during clinical trials and were found to be critical point in development of paclitaxel. Paclitaxel was approved for phase II trials in April 1985, only after including premedication with antihistamines in the regimen along with 24 h continuous infusion to reduce peak concentration of both Cremophor and taxol.

2.2.5. Alternative formulations of paclitaxel for potential clinical applications

Paclitaxel has been recognized as the most potent anticancer agent for the past few decades. However, its use as an anti-cancer drug is compromised by its intrinsically poor water solubility. The effective chemotherapy using paclitaxel is relying on the development of new delivery systems which attracted a substantial number of studies investigated to deliver paclitaxel by new formulations.

The primary goal of formulation development for paclitaxel is to eliminate the Cremophor vehicle by reformulation of the drug in a better-tolerated vehicle which

has the possibility of improving the efficacy of paclitaxel based anticancer therapy. A great deal of effort is being directed towards the development of aqueous based formulations for paclitaxel, including soluble semi-synthetic paclitaxel derivatives that do not require solubilization by Cremophor and that decrease the systemic clearance of the drug (Singla et al., 2002).

Some of these efforts to achieve a safer and better-tolerated formulation include water soluble prodrugs (Greenwald et al., 1996; Pendri et al., 1998), enzyme activatable prodrugs used in conjugation with antibodies (Rodrigues et al., 1995), albumin conjugates (Dosio et al., 1997), complexes with cyclodextrins (Sharma et al., 1995; Dordunoo and Burt, 1996) and conjugates (Li et al., 1998), parenteral emulsions (Tarr et al., 1987; Simamora et al., 1998; Kan et al., 1999), nanocapsules (Bartoli et al., 1990), liposomes (Sharma et al., 1996a), mixed micelles (Chai et al., 1994), and micro- and nanoparticles of biodegradable polymers as controlled drug delivery systems.

Among these, some dosage forms have been able to dissolve substantial amounts of paclitaxel and successfully improved the effects of anti-tumor activity in animal models. Water miscible co-solvents are used as the method of formulating intravenous non-water-soluble drugs. Paclitaxel, inherently possessing limited aqueous solubility can be rendered water soluble by using principle of co-solvency (Tarr and Yalkowsky, 1987; Bissery et al., 1991), commonly used solvents being polysorbate 80, ethanol etc. However, the precipitation of the drug on dilution with the aqueous media is an important factor to be considered.

In order to improve solubility while preserving the activity, a number of water soluble paclitaxel prodrugs and derivatives have been synthesized that contain hydrophilic or

charged functionalities attached to the specific sites on the paclitaxel molecule (Hayashi et al., 2003). Sugars can be used as a hydrophilic appendage to tether them to the C-10 hydroxyl group in a taxoid through an ester linkage, since the taxoid is susceptible to Lewis acids often utilized in the conventional glycosylation protocols.

Prodrug synthesis has also been extensively studied to increase the aqueous solubility of paclitaxel (Burt et al., 1995). The preferred position for the preparation of prodrug of paclitaxel is 2' position since many 2'-acyl-paclitaxel derivatives hydrolyze fairly rapidly back to paclitaxel in blood compartments (Mellado et al., 1984). Since the configuration of C-7 hydroxyl group does not seem to be a factor in determining cytotoxicity, C-7 prodrug ester has also been synthesized (Deutsch et al., 1989). In vitro, these prodrugs have been shown to possess cytotoxic activity against tumor cell lines comparable to those of paclitaxel. In addition, human plasma catalyzes the release of active paclitaxel. A prodrug strategy employing PEG as a solubilising agent has been successfully demonstrated in case of paclitaxel (Greenwald et al., 1994, 1996).

New paclitaxel amino acid derivatives were synthesized which have a glutaryl group substituted at the 2' position followed by the reaction of a peptide link between the carboxyl and the amino terminal group of the amino acid (Paradis and Page, 1998). The derivatives were cytotoxic in vitro against many sensitive cell lines. They also increased G2+M phase arrest. These derivatives were stable for over a year and showed a better solubility in water than the parent compound. However, no derivative has progressed to clinical evaluation because of problems such as chemical instability and the loss of pharmacological activity.

Several approaches were investigated to improve oral bioavailability of paclitaxel. These include the association of the drug complex to natural or modified cyclodextrins and to colloidal drug carrier systems, particularly polymeric nanoparticles (Boudad et al., 2001) which have proved to be an effective controlled drug delivery system.

However, problems have still been encountered with these alternative methods, such as in vivo stability of liposomes and dosage limiting toxicity of the dosage forms used (Tarr et al., 1987; Sharma et al., 1995). The possible leakage of the drug from the liposomes formulation constrained the successful development of formulation using liposome which is a promising drug carrier. The mixed micelles formulation and parenteral emulsions have the similar safety problems to be resolved. New formulations with more consistent release properties are necessary to be developed for successful delivery of paclitaxel to human body.

Polymer micelles are convenient passive targeting carrier systems of anticancer drugs since they are structurally strong and unlike liposomes are not captured by the reticuloendothelial cell system (RES) because of their particle size (20–100 nm) (Yokoyama et al., 1990; Kataoka et al., 1993). Polymeric micellar paclitaxel formulation using non-toxic, biodegradable polymers have also been developed (Ramaswamy et al., 1997; Miwa et al., 1998). Using panel of cell cultured lines; it was found that the cytotoxic activity of paclitaxel was retained when formulated as mixed-micellar solution. In addition, for the same solubilization potential, the mixed-micellar vehicle appeared to be less toxic than the standard non-aqueous vehicle of paclitaxel containing Cremophor EL (Chai et al., 1994). Micelle encapsulated

paclitaxel is thereby water soluble and in addition devoid of common side effects associated with Cremophor vehicle.

None of the methods seemed to be effective enough to replace CrEL based vehicle although the different approaches investigated so far have shown a lot of promise for paclitaxel delivery. The final product for human use is still far away. For that reason, a great deal of effort has been given to develop more tolerable vehicles that improve the efficacy of paclitaxel in clinical chemotherapy.

Of these alternatives solutions, nanoparticles and microparticles attained much importance, mainly due to their tendency to be able to accumulate in inflamed areas of the body (Diepold et al., 1989; Illum et al., 1989; Alpar et al., 1989). Moreover, they possess better stability in biological fluids and during storage.

Clinical trials showed satisfactory results (Ichihara et al., 1989, Wang et al., 1993) and thus, the use of nanoparticles or microparticles of biodegradable polymers for chemoembolization has been pursued in efforts to achieve the desired result of enhancing the therapeutic efficacy of anticancer agents while minimizing its systemic order effects.

The current approaches are mainly focused on developing formulations that are devoid of CrEL, the possibilities of preparation on a large scale and stability for longer periods of time (Panchagnula, 1998; Feng et al., 2000; Feng and Huang, 2001; Mu and Feng, 2001; Wang et al., 2002). Various anticancer drugs such as cisplatin, interferon β, etc. can be encapsulated together to achieve synergistic effects (Zand et al., 2001).

2.2.6. Our engineering approach for potential alternative clinical paclitaxel formulation

There has been intensive investigation directed to oral delivery of paclitaxel, which is expected to provide a long-time exposure at an appropriate therapeutic level of drug and to greatly improve the quality of life of the patients. However, the obstacle to the successful formulation of oral dosage form is its low oral bioavailability due to the elimination of a multi-drug efflux pump transporter P-glycoprotein (P-gp) (Sparreboom et al., 1997) and the first pass of cytochrome P450 (CYP 3A) enzymes (Cresteil et al., 1994; Harris et al., 1994). P-gp in the mucosa of the small and large intestine may limit the oral uptake of paclitaxel and mediate direct excretion of the drug in the intestinal lumen (Adams et al., 1993). Medical solution to overcome this problem is to apply P450/P-gp inhibitors such as cyclosporin A (Scambia et al., 1995; Asperen et al., 1997; Bardelmeijer et al., 2000; Choi et al., 2004). However, the inhibitors would also fail the immune system of the patients and thus lead to medical complications over long term application. Most of the P450/P-gp inhibitors have their own side effects and difficulties in formulation (Bonduelle et al., 1996). Moreover, the cost of these inhibitors is another hindrance for successful development of oral dosage form of paclitaxel.

Nanoparticles of biodegradable polymers represent a chemotherapeutic engineering solution or cancer nanotechnology solution. Nanoparticles of biodegradable polymers for drug formulation and for oral chemotherapy have shown advantages in improving the pharmacokinetics and tissue distribution and thus the therapeutic effects of the formulated drug (Couvreur et at., 1980; Rolland, 1989). Preliminary results have demonstrated that nanoparticles can escape from the vasculature through the leaky

endothelial tissue that surrounds the tumor and thus accumulate in solid tumors (Leroux et al., 1996; Monsky et al., 1999). It was demonstrated that nanoparticle formulation can overcome the multi-drug resistance phenotype mediated by P-glycoprotein and thus lead to an increase in drug content inside the neoplastic cells (Benis et al., 1994; Rowinsky and Donehower, 1995; Seelig, 1998).

2.3. Biodegradable Polymeric Nanoparticles as Controlled Drug Delivery Systems

Polymeric drug delivery systems have been a major focus of pharmaceutical companies in recent years (Sandor et al., 2001). The controlled release of pharmacologically active agents to the specific site of action at the therapeutically optimal rate and dose regimen has been a major goal in designing such delivery systems. Liposomes have been used as potential drug carriers instead of conventional dosage forms because of their unique advantages such as ability to protect drugs from degradation, target the drug to the site of action and reduce the toxicity or side effects (Knight, 1981). However, liposomes formulation has some inherent limitations such as low encapsulation efficiency, rapid leakage of water-soluble drug in the presence of blood components and poor storage stability. Polymeric drug delivery devices offer some specific advantages over liposomes. They help to increase the stability of drugs /proteins and possess useful controlled release properties.

The advantages of biodegradable and biocompatible polymeric drug delivery devices (Gardner, 1987; Sandor et al., 2001) over traditional dosage forms include: (1) improved therapeutic efficacy and reduced toxicity; (2) lower and more efficient doses; (3) less frequent dosing; (4) better patient compliance; (5) the ability to

stabilize drugs and protect against hydrolytic or enzymatic degradation; (6) the elimination of the need for surgical removal of the devices; and (7) the ability to mask unpleasant taste or odor (if any).

The major advantage of polymeric drug delivery system is that the drug in the polymer matrix is unaltered, therefore its biological effect, absorption, distribution, metabolism, and excretion after being released from the polymer is the same as that of the native drug (Dunn, 1991). Moreover, the release profile can be modulated by controlling the proper parameters when the system is prepared.

Over the past few decades, there has been considerable interest in developing biodegradable nanoparticles as effective drug delivery devices, owing to their ability to target particular organs/tissues, act as carriers of DNA in gene therapy, and deliver proteins, peptides through a peroral route of administration (Lanza et al., 1997; Langer, 2000). Nanoparticles (NPs) were first developed around 1970 and initially devised as carriers for vaccines and anticancer drugs (Couvreur et al., 1982). The drug can be dissolved, entrapped, encapsulated or attached to NP matrix. One of the simplest methods to obtain sustained delivery of a biologically active agent is to encapsulate or entrap within the polymer. The polymer has to dissolve or disintegrate before the drug can be released or else the drug has to dissolve or diffuse from the polymer matrix. In either case, the release of drug to the physiological environment is extended over a much longer time than if the drug is administered in its native form.

Furthermore, the strategy of drug targeting was employed to enhance tumor uptake. This important research focused on the development of methods to reduce the uptake of the nanoparticles by the cells of the reticuloendothelial system (RES) (Couvreur et

al., 1986). The use of nanoparticles for ophthalmic and oral delivery was also investigated (Labhasetwar et al., 1997).

Depending on the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. Nanoparticles generally vary in size from 10 to 1000 nm.

Nanoparticles present some significant advantages: nanoparticles enable intravenous injections and intramuscular and subcutaneous administrations due to the reduction of possible irritant reactions involved and are also capable of avoiding uptake by microphages (Oppenheim et al., 1982; Aprahamian et al., 1987; Florence, 1997). Moreover, due to the small size of these polymeric nanoparticles, the encapsulated anticancer drug can escape the elimination by P-gp present in the intestinal cell membrane and/or enter between the intestinal cells.

Nanoparticles have very special properties compared with bulk materials or even micron-size particles. Due to its sub-micron nature, it is more efficient in certain therapy applications such as intracellular localization of therapeutic agents (Labhasetwar et al., 1997). Nanoparticles enable intravenous administration by minimizing possible irritant reactions. Nanoparticles could prove to have potential applications in oral drug delivery due to their efficient uptake by the gut associated lymphatic tissue (GALT), and to improve oral bioavailability of therapeutic agents (Desai et al., 1996). Moreover, oral administration of nanoparticles encapsulated agent could provide sustained efficacy due to slow breakdown of the polymer over a period of time.

Oral absorption of polymeric nano- and microparticles by the GI tract has been extensively studied for the last two decades. The controlling factors are found to be size, nature of the polymer, zeta potential, vehicle, coatings, and presence of nutrients. However, the best designed parameters for oral delivery system can not be determined yet due to numerous discrepancies lying in the large variety of analytical methods and models employed to investigate the particle uptake.

2.3.1. Polymeric delivery system formulation for paclitaxel

The use of a biodegradable delivery system capable of providing a lower and localized dose of paclitaxel to the tumor site attracted intense research on its formulation and applications. Several researchers have examined the release of paclitaxel and other radiosensitizers from a variety of different carriers (microspheres, discs, nanoparticles) using poly (lactide), poly (glycolide) and other co-polymers (Doiron et al., 1999).

The low solubility of paclitaxel may remain as a barrier to design a successful formulation with desired sustained release property, even being encapsulated into the nanoparticle matrix. Paclitaxel molecules are homogeneously distributed in the solid polymer matrix and are not readily dissolved and diffused out of the polymer solid phase due to its poor solubility and the very limited amount of water available inside the solid phase. Various approaches have been attempted to solve this problem. Isopropyl myristate, a fatty acid ester, was added to the solid polymer microparticles and the release rate of paclitaxel was increased about 3 times higher than the blank samples (Wang et al., 1997). However, the release time span of this formulation was too short for a radiotherapy or chemotherapy which usually lasts for about 6-8 weeks.

Polymeric paste formulation was investigated using low molecular weight poly (D, L-lactic acid) (PLA) and polycaprolactone (PCL) as additives to modulate the release profile of paclitaxel from the poly (D, L-lactic acid)-block-poly (ethylene glycol)-block-poly (D, L-lactide) (PDLLA-PEG-PDLLA) copolymer paste (Zhang et al. 1996). Both the addition of low molecular PLA and the use of copolymer PDLLA-PEG-PDLLA increased the release rate of the drug comparing with the samples made of PCL. However, the copolymer needs to be approved by FDA for the use in human body as a biocompatible material and the high temperature used in the experiment may cause the degradation or other side reaction of the drug. The effects of various water soluble additives such as gelatin, bovine serum albumin, methylcellulose, and dextran T500 on the enhanced release of paclitaxel from a polymeric surgical paste was studied (Dordunoo et al., 1996). All the additives increased the release rate by a possible swelling and solubilizing mechanism.

Significant amount of works has been done by examining various aspects, such as influence of polymer type, concentration, particle formation conditions, and drug type on the resultant properties of the controlled release system.

2.3.2. Biodegradable polymers

Drug delivery systems that control and prolong the action of therapeutic agents have grown in importance during the recent years with the development of biodegradable polymers. The use of biodegradable polymers eliminates the step of removing the system after the drug has been released, which, in some applications, can represent a significant advantage over other systems. Much of the effort on encapsulation of drug in the pharmaceutical field has concentrated on using biodegradable polymers.

Biodegradable polymers are gradually dissolved by hydrolytic or enzymatic cleavage of the polymeric structure or by simple dissolution. Generally, there are three defined mechanisms of polymer erosion (J Heller, Biomaterials, 1, 51-57). Mechanism I concerns polymers that are made water insoluble by their hydrolytically unstable cross-links. These polymers, such as cross-linked gelatin, collagen, or poly (vinyl alcohol), provide highly hydrophilic matrices. Thus, substances with low molecular weight and high water solubility diffuse rapidly through polymeric network independently of the rate of erosion of the matrix. These polymers are mainly used for the release of sparingly water soluble drugs and for macromolecules such as enzymes and antigens.

Mechanism II includes polymers that are initially water insoluble and are solubilized by hydrolysis, ionization, or protonation of a pendent group yet lack backbone cleavage. This mechanism has the feature of not causing any significant change in the molecular weight of the polymer. These polymers, such as poly (vinyl methyl ether/maleic anhydride), cannot therefore be used for implants because of the difficulty of their elimination.

Mechanism III refers to hydrophobic polymers that are converted to small soluble molecules by backbone cleavage. As long as these breakdown products are not toxic, these polymers, for example, polyanhydrides, polycaprolactones, poly (ortho ester)s, poly (lactic acid)s (PLA), poly (glycolic acid)s (PGA), and their copolymers, are suitable as implantable carriers for the administration of drugs to any organ. They offer a wide range of applications in the ophthalmic field, whether applied on the surface of the eye or as intraocular implants.

The breakdown of polymers does not necessarily proceed through one mechanism only, and erosion occurs by a combination of these mechanisms.

A broad range of polymers can be used to form micro/nanoparticles but the list of polymers approved for use in oral and/or parenteral drug formulation is limited. This list includes proteins, polysaccharides, cellulose derivatives, synthetic polyesters developed as synthetic suture materials, polyanhydrides, and polyphosphazene.

Various polymers have been used in drug delivery research as they can effectively deliver the drug to a target site and thus increase the therapeutic benefit, while minimizing side effects (Kreuter, 1994). The biocompatible polymers such as PLA, PLG and PLGA have been used as controlled release formulations in parentral and implantation drug delivery applications (Wise et al., 1979). In addition, poly (ε-caprolactone), PCL, which was first reported by Pitt's group (1979 and 1980) for the controlled release of steroids and narcotic antagonists as well as to deliver ophthalmic drugs (Calvo et al., 1996), and poly (alkylcyanoacrylate), PACA, are now being developed as NPs.

Polymers with the greatest relative hydrophobicity, poly (styrene), poly (methyl methacrylate) and poly (hydroxybutyrate) were absorbed most readily. The relatively less hydrophobic polymers poly (D, L-lactide), poly (L-lactide) and poly (D, L-lactide-co-glycolide) were absorbed well, but in low numbers. Based on the criteria of biocompatibility, biodegradability, adequate absorption by the Peyer's patches and a history of safe use in humans, particles prepared with DL-PLGA were selected as the most promising for application as an oral delivery system.

2.3.2.1. Poly (lactide-co-glycolide) (PLGA)

Initially, poly(α-hydroxy acid)s were developed as synthetic, bioresorbable sutures in the 1960s. As a result of their good histocompatibility (Kobayashi et al., 1992), this family of polyesters now includes the most-investigated biodegradable polymeric carriers for drug delivery systems. The principal polymers of this class are homo- and co-polymers derived from lactic and glycolic acids: poly-lactide (PLA), poly-glycolide (PGA), and their co-polymer poly-(lactide-co-glycolide) (PLGA).

Poly (lactide-co-glycolide) (PLGA) is a type of thermoplastic aliphatic and hydrophobic poly (ester). As the name suggests, PLGA is prepared from the co-polymerization of D, L-lactide with glycolide. The chemical structure of PLGA is shown in Fig. 2.2.

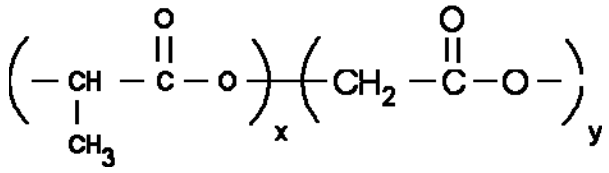


Figure 2. 2. Structure of PLGA. The suffixes x and y represent the number of lactic and glycolic acid respectively.

Poly(α-hydroxy acids) are generally synthesized by a condensation reaction at high temperatures. The ring-opening polymerization of cyclic glycolic and lactic acid diesters is an efficient method to produce high-molecular-weight polymers. The simple step-growth polymerization method leads only to low-molecular-weight polymers with poor mechanical properties (Vert et al., 1984). The presence of an asymmetric carbon in lactic acid makes it possible to obtain levorotatory (L),

dextrorotatory (D), or racemic (D,L) forms of the corresponding polymer, which can have different physicochemical properties.

PLGA is believed to degrade in vivo by nonspecific hydrolytic scission of the ester bonds to yield the monomeric units of lactic acid and glycolic acid (Brandt et al., 1984) which are eventually excreted from the body. Therefore, it is considered as a biodegradable and biologically friendly polymer. Precise specification of PLGA drug delivery system can be tailored by selecting the required ratio of LA/GA and molecular weight which control the degradation and drug release profiles of a PLGA delivery system.

The physical properties and FDA approval of PLGA make them the most extensively studied commercially available biodegradable polymers. These copolymers are amorphous and easily dissolve in organic solvents such as dichloromethane and ethyl acetate. The degradation rate of PLGA in water is a function of the molecular weight and the lactide:glycolide ratio. Higher glycolide content and lower molecular weight increase the degradation rate. PLGA degrades by bulk hydrolysis in water (Gopferich, 1997). The rate of degradation of PLGA controls the release of any encapsulated pharmaceutical agent which is released by both diffusion and erosion as the polymer hydrolyzes. Thus, depending on the therapy required for a particular drug, the release kinetics of that drug from its polymer matrix can be controlled by selecting a PLGA with appropriate physical characteristics.

The properties of PLGA, biodegradability and low toxicity, make it suitable to be used in various medical and pharmaceutical applications such as wound closure, dental repairs, fracture fixation, ligament reconstruction, vascular grafts, tracheal replacement, ventral herniorrhaphy, nerve repair and drug delivery (Wu, 1995).

PLGA degrades into lactic and glycolic acids. The lactic acid enters the tricarboxylic acid cycle and is subsequently eliminated from the body as carbon dioxide and water. Glycolic acid is either excreted unchanged in the kidneys or it enters the tricarboxylic acid cycle and is eliminated from the body as carbon dioxide and water (Jain, 2000). The greatest advantage in using PLGA in drug delivery lies in the fact that they can be degraded into biologically accepted molecules that are metabolized and removed from the body via normal metabolic pathways (Peppas, 1997).

2.3.3. Fabrication of nanoparticles for drug delivery system

Several methods are available in the literature to formulate nanoparticles with drugs dispersed uniformly into the polymer matrix or simply adsorbed onto preformed particle surface. Some processes involve polymerization reactions while others use the preformed polymers. The choice of polymer and method depends on the desired properties of the final product (morphology, release profiles, etc.), the properties and nature of the polymer and the drug, and the properties of the coating material.

There are a huge and increasing number of particle preparation processes such as: interfacial polymerization, coacervation, complex coacervation, thermal denaturation, salting-out, hot melt, solvent evaporation, solvent removal, spray-drying and phase separation. It is sometimes difficult to classify the methods because specific techniques can be hybrids of two or more methods or can use different mechanisms simultaneously.

Interfacial polymerization involves the condensation of two monomers at the interface of the organic and aqueous phases. Monomer polymerization is employed to prepare drug-loaded nanoparticles. The drug can be dissolved in the polymerization

medium before the addition of the monomer or at the end of the polymerization reaction. The formed nanosuspension is then purified by ultracentrifugation or by resuspending in an isotonic medium. The size and molecular mass of obtained nanoparticles depends on the polymerization variables, such as the type and concentration of the surfactant used, the pH of the polymerization medium, the concentration of monomer and the stirring speed (Gaspar et al., 1991; Behan et al., 2001).

Coacervation or **phase separation** process uses the common phenomenon of polymer-polymer incompatibility to form nanoparticles. The particle wall material polymer is dissolved in a solvent and a second polymer (the phase inducer) is introduced to form two polymer-rich phases. If drug particles are then introduced, one phase, rich in the desired coating polymer, engulfs the drug being encapsulated.

Coacervation can be induced by a change in temperature or pH of the system; a change in electrolyte balance; addition of nonsolvents; addition of other materials which are incompatible with the polymer solution. Phase separation is a new method in which a one-step precipitation of two polymers or more produces double-walled microspheres (Pekarek et al. 1994). Although the range of polymers that can be used in this process is essentially infinite in principle, the number of polymers that have been used successfully in practice is relatively small.

The term coacervation used in this context is borrowed from the description of an equilibrium state of colloidal systems. In the phase separation process, the term refers to a state of partial precipitation of the matrix phase by the coacervation agent. The matrix material at this point is still solvent rich, and not completely precipitated, and thus maintains a high degree of flow necessary for good film- and particle-forming

properties. Several of the coacervation methods are being successfully used to prepare a variety of products from peptide microcapsules to encapsulated electrolytes (Migliaresi et al., 1987).

Complex coacervation was used to make the microcapsules in the first successful encapsulated product, carbonless copy paper (Kassem and El-Sayed, 1975). This process uses the interaction of two oppositely charged polyelectrolytes in water to form a polymer-rich coating solution called a coacervate (Chan and Heng, 1998). This solution (or coacervate) engulfs the liquid or solid being encapsulated, thereby forming an embryo capsule. Cooling the system causes the coacervate (or coating solution) to gel via network formation. Gelatin is a primary component of most complex coacervation systems.

The **precipitation** and/or **gelation** process precipitates a preformed polymer around the core (or a multi- particulate core) to encapsulate the drug in the particles formed. The polymer is dissolved in water miscible organic solvent and the drug is dissolved or dispersed in the polymer solution which is then poured to water phase in the presence or absence of a surfactant. The interfacial turbulence produced by the quick diffusion of solvent to the external phase leads to the formation of nanoparticles. One example is the precipitation of water-soluble polymers such as gelatin with water-miscible solvents such as isopropanol. Other examples include the precipitation of ethyl cellulose from cyclohexane by cooling, the gelation of sodium alginate with aqueous calcium salt solutions (Chan and Heng, 1998), and the thermally induced precipitation of proteins to form microspheres.

Salting-out process involves the addition of salt to an aqueous polymer solution ultimately causing the polymer to phase separate from solution (Allémann et al.,

1992). One potential problem with this process is the possibility of incorporating a relatively high concentration of salt in the final product, as these salts may have an adverse effect on the drug release behavior.

The nano or microparticles are usually prepared by the solvent evaporation technique, either by freeze dry or spray dry (Burt et al., 1995; Wang et al., 1996; Wang et al., 1997; Corrigan et al., 1985; Giunchedi et al., 1995). In spray-drying, the evaporation of the solvent is achieved in a special, temperature-controlled cyclone.

The **emulsification-solvent-evaporation** technique was first described by Beck and colleagues for the delivery of contraceptive steroids (Beck et al., 1979). It is the most commonly used method for preparation of biodegradable micro- or nano-particles encapsulating poorly soluble therapeutic agents. The polymer and drug are dissolved in an organic solvent which is volatile and immiscible with water. A dispersed phase containing the polymer is emulsified in an immiscible continuous phase containing a stabilizing agent. The second phase involves the diffusion of the solvent from the emulsion droplet into the continuous phase and its subsequent evaporation. Simultaneous inward diffusion of the non-solvent into the droplet causes polymer precipitation, microsphere formation, and hardening. Depending on the nature of the two phases, the process may be termed oil-in-water (o/w) or water-in-oil (w/o) emulsion method.

The solvent evaporation process requires the use of a surfactant or emulsifier to stabilize the dispersed-phase droplets formed during emulsification and inhibit coalescence. Surfactants are amphipathic in nature and hence align themselves at the droplet surface to promote stability by lowering the free energy at the interface between the two phases. Moreover, the creation of a charge or steric barrier at the

droplet surface confers resistance to coalescence and flocculation. Surfactants employed in the process tend to be hydrophilic in nature and by far poly (vinyl alcohol) (PVA) is the most widely used. The amount and type of emulsifier used play a significant role in determining morphology, size, encapsulation efficiency, release profile and particle uptake. For pharmaceutical applications, emulsifiers must be acceptable for therapeutic use (Wade and Weller, 1994).

Particle size can be adjusted by the source and the energy input such as sonication, homogenization, microfluidization, vortexing, etc. for emulsification in the system (Desai et al., 1997; Labhasetwar et al., 1997).

After emulsification, the removal of solvent and the complete particle hardening is usually accomplished by gentle agitation of the suspension. After evaporation of the solvent, the final stage of the emulsification-solvent- evaporation process is the isolation of particles from the dispersed phase containing surfactant. This has generally been achieved by centrifugation and filtration, and it is usually followed by a further cleaning process in which the particles are washed several times with distilled water. The particles are finally dried at ambient conditions or under vacuum, or using freeze-drying or fluid-bed drying.

One disadvantage of using (o/w) emulsification method is poor encapsulation efficiency for moderately water-soluble and water-soluble drugs. For the encapsulation of hydrophilic drug, a modified method of oil-in-oil is used. In this method, an organic liquid such as mineral or castor oil is used as the continuous phase. An alternative method involves forming a double emulsion where an aqueous drug solution is emulsified in a polymer solution in volatile organic solvent. The

resulting emulsion is then emulsified in an aqueous solution giving a double emulsion.

Although the solvent evaporation method is simple, various parameters will influence the formation of the particles. These include the polymer composition and molecular weight, the nature and solubility of the drug encapsulated, the temperature of the emulsion and the properties of the emulsifiers used.

Solvent extraction is similar in nature to o/w emulsion-solvent evaporation process except that following emulsification the preformed particles are poured into a large volume of non-solvent to extract the remaining organic solvent, thus causing rapid hardening of the particles.

Solvent removal was developed as a modification of the solvent evaporation technique, using organic solvents as the extracting medium (Mathiowitz et al., 1988).

Spray-drying is a one-step, continuous, particle-drying process that transforms the feed material from a fluid state into a dried particulate form by spraying the feed into a hot drying medium. The feed material can be in the form of a solution, suspension, emulsion, or paste. The resulting product can be powdered, granular, or agglomerated particles, depending upon the physical and chemical properties of the feed material, the drier design, and its operation. Spray-drying is used in all major industries where particle drying is required, ranging from food and pharmaceutical manufacturing to chemical industries such as mineral ores and clays (Masters, 1984). Spray-drying has proven to be a useful technique in producing controlled release products. The main advantage of this process is that it is convenient, fast, and allows the use of mild conditions.

2.3.4. Characterization of polymeric nanoparticles

Among the various techniques and instrument available, the characterization of particles employed laser light scattering (LLS) for measurement of size and size distribution, atomic force microscopy (AFM) and scanning electron microscopy (SEM) for morphological studies, X-Ray photoelectron spectroscopy (XPS) for surface chemistry, zeta potential for surface charge and stability of nanoparticle suspension, and high performance liquid chromatography (HPLC) to study the encapsulation efficiency and the in vitro release of the drug. The instruments used and the working principles behind the instruments are briefly described in this section.

2.3.4.1. Laser light scattering system (LLS)

The laser light scattering instrument is used to determine the mean diameter and polydispersity of the sample particles. The dried powder samples are suspended in filtered deionized water. The scattering of the laser beam focused on this solution/suspension containing particles of interest will provide information about their molecular structure and motion in the solution.

Interaction of light (electron-magnetic radiation) with matter results in two processes, absorption and scattering. When a beam of light falls upon a matter, the electric field associate with the light polarizes the electron cloud of the atoms. Thus a dipole is induced in the molecules, which oscillates with the electric field. These oscillating electron clouds serve as secondary sources of light and emit light in various directions (scattering). The scattered light has almost the same wavelength as the incident light. It is the intensity of the scattered light and its angular distribution which gives the information regarding the nature of the molecule under study.

Various applications of the instrument include characterization of macromolecules and their associations, determination of macromolecular size, translation diffusion coefficient and the polydispersity of the sample.

2.3.4.2. Scanning Electron Microscopy (SEM)

The SEM is used to study shape and morphology of the sample particles. The samples are coated with platinum or gold before analysis by SEM. An electron beam is focused onto the sample surface kept in a vacuum by electron-magnetic lenses and scanned over it. The scattered electron from the sample is fed to the detector and then to a cathode ray tube through an amplifier, where the images are formed, which gives the information on the surface of the sample. The instrumentation comprises of heated filament as source of electron beam, condenser lenses, aperture, and evacuated chamber for placing the sample, electron detector, amplifier and CTR with image forming electronics.

While conventional light microscope uses a series of glass lens to bend light waves and create a magnified image, the scanning electron microscopy (SEM) uses electrons instead of light. The SEM is capable of producing very detailed 3-dimensional images at much higher magnifications than is possible with a light microscope.

SEM has been used in the surface studies of materials such as metals, polymers and biological materials. Some of the disadvantages of the SEM are complex instrumentation and the requirement of high vacuum for optimum performance.

2.3.4.3. Atomic force microscopy (AFM)

The AFM is a form of scanning probe microscopy, which provides the information on morphology and properties of surfaces on a nano scale range. AFM can be used to study the surface topography, surface hardness and elastic modulus.

AFM operates by measuring the forces between the sample and the tip. The probe tip is brought close enough to the sample surface to detect the repulsive force between the atoms of the tip material and sample. The probe tip is mounted at the end of a cantilever of a low spring constant and the tip-to-sample spacing is held fixed by maintaining a constant and very low force on the cantilever. If the tip is brought close to the sample surface, the repulsive force will induce a bending of the cantilever. The bending of the cantilever can be detected by a laser beam which is reflected off the back of a cantilever. The surface topography of the sample can be tracked by monitoring the deflection of the cantilever. The instrumentation for AFM consists of a scanner, cantilever, laser source, photodiode detector and microprobe.

AFM operates at two modes, contact mode that detects the repulsive forces between the tip and sample or non-contact mode that detects the van der waals forces that act between the tip and sample. The AFM has proved to be a most versatile tool in imaging of surfaces and molecules and to measure forces between nanoparticles as a function of separation.

2.3.4.4. X-Ray Photo-emission Spectrometry (XPS)

The XPS is used to analyze the surface chemistry of the particles. When a primary X-ray beam of precisely known energy impinges on sample atoms, inner shell electrons

are ejected and the energy of the ejected electrons is measured. The difference in the energy of the impinging X-ray and the ejected electrons gives the binding energy of the electron to the atom. The binding energy of the emitted electron depends on the energy of the electronic orbit and the element, thus it can be used to identify the element involved. The chemical form of the atom affects the binding energy to a considerable extent to give rise to some chemical shift, which can be used to identify the valence of the atom and its exact chemical form.

Instrumentation for XPS consists of a radiation source for primary X-rays, monochromator, energy analyzer and detected to measure the intensity of the resolved electrons. The analysis is carried out in high vacuum. Some of the disadvantages associated with the use of XPS are complex instrumentation and the inability of XPS to detect impurities at ppm or ppb level.

2.3.4.5. Zeta Potential Analyzer

Zeta potential is an important indicator of the electronic charge on the surfaces of the microscopic material, which can be used to predict and control the stability of colloid suspensions or emulsions. The zeta potential of the sample particles suspended in filtered deionized water was measured by tracking the charged particles when they migrate in a voltage field in a zeta potential analyzer.

2.3.5. *In vitro* evaluation by cell line models

Cells taken from animal/human tissue will continue to grow by mitosis if supplied with nutrients and growth factors and this cell culture process occurs *in vitro* as opposed to *in vivo*. Cultures normally contain cells of one type (e.g., fibroblasts). The

cells are selected and maintained by sub-culturing or passaging as independent units. The cells in the culture may be genetically identical (homogeneous population) or may show some genetic variation (heterogeneous population).

The culture collections provide the cells that have been well characterized in terms of growth, origin, and genetic traits. The choice of cell culture depends on the objective of the study and the nature of the experiments planned. Most cells in culture grow best at 37°C and pH 7.4 which are the normal physiological conditions.

In vitro models are expected to give better reproducibility and to allow screening of higher number of samples compared to *in vivo* models. There are several advantages (Butler, 1996) of using cell line experiments: the consistency and reproducibility; the understanding of the behavior of cells in vivo and the effects of a particular compound on a specific cell type such as lung cells or intestinal cells; the rapid assessment of the potential permeability and metabolism of a drug; the opportunity to elucidate the molecular mechanism of drug transport; the opportunity to use human rather than animal tissues; and the opportunity to minimize time-consuming, expensive, and sometimes controversial animal studies. Cell line experiments are highly preferred and appreciated as an initial study to understand and/or predict in vivo conditions.

In vitro cell culture models allow both quantitation and visualization of particle transport and localization on a cellular level using a noninvasive technique. Moreover, cell integrity and cytotoxicity can be assessed (Jung et al., 2000) which are especially important parameters of intracellular uptake since toxic side effects of the carrier system are known to increase epithelial permeability and can lead to misinterpretations. Fluorescent particles are the most common tools to assess particle uptake both qualitatively and quantitatively.

Confocal microscopy and fluorescence microscopy are widely applied for qualitative uptake studies. Laser scanning confocal microscopy is an established valuable tool for high resolution images and 3-D reconstructions of a variety of biological specimens. Desai and colleagues (1997), Suh and co-workers (1998) have extensively used confocal microscopy to evidence the fluorescent particle uptake and the extent of uptake.

Human colon adenocarcinoma cell lines, Caco-2 and HT-29, established by Fogh and co-workers (1975) have received a great deal of attention in recent years because of their ability to express morphologic features of mature enterocytes or goblet cells. P-gp mediated drug transport *in vitro* has been successfully studied using Caco-2 cells (Artursson et al., 1996; Quaroni and Hochman, 1996).

Both Caco-2 and HT-29 cells have been widely utilized as in vitro tools for the study of intestinal epithelial differentiation, function, and uptake, binding and transport phenomena of drugs and nutrients (Audus et al., 1990; Desai et al., 1997; Walle and Walle, 1998; McClean et al., 1998; Russell-Jones et al., 1999; Kamm et al., 2000; Jung et al., 2000; Ho and Storch, 2001; Yamaguchi et al., 2001). Caco-2 cell line is a more relevant in vitro model than HT-29 cell line since Caco-2 cells exhibit morphological and functional similarities to intestinal (absorptive) enterocytes, possess a higher degree of enterocytic differentiation and spontaneous dome formation (Audus et al., 1990; Artursson et al., 2001), perform most of the functions associated with intestinal lipid metabolism, transport, and metabolism (Ho and Storch, 2001).

2.3.5.1. Studies of transport processes

Recent studies in epithelial cell monolayers have developed understandings of the functions of a variety of transport mechanisms of endogenous as well as exogenous molecules such as drugs. The availability of tissue culture-treated microporous membranes facilitates the cell line model to represent a more physiologic environment because it allows the exchange of substances across both the apical and the basolateral membranes (Audus et al., 1990). Proper selection of filter material, filter diameter and pore size is important depending on the particular application. The integrity of the cell monolayer can be determined by measuring transepithelial electrical resistance (TEER) using a volt/ohmmeter.

Only a small quantity (< 1 %) of molecules that survive degradation in the gut is able to cross the epithelial cell layer and enter the circulation. The coating of particles with targeting agent molecules such as lectins may enhance their binding to intestinal epithelial cells (Russell-Jones et al., 1999) to stimulate the enterocytes to endocytose the coated particles and transport the particles across the cell and into the circulation, thus improving oral bioavailability (Naisbett et al., 1993; Florence et al., 1995; Hussain et al., 1997; Russell-Jones et al., 1999).

Tomato lectin has also been reported to cross the tissue of rat everted intestinal sacs in vitro by a mechanism involving adsorptive endocytosis (Naisbett et al., 1993). However, Lehr and Lee (1993) did not observe significantly enhanced transport of bioadhesive lectins due to adsorptive endo-/transcytosis in comparison with the transport of a nonadhesive protein (albumin) by Caco-2 cell line and fluid phase transcytosis.

In a recent study by Russell-Jones and co-workers (1999) using Caco-2 cells and opossum kidney carcinoma cell line (OK cells), a range of targeting molecules, including LTB (the binding subunit of E. coli heat labile toxin), ConA (which binds to ∝-D-mannose) and WGA (wheat germ agglutinin) are reported to be suitable for delivery of nanoparticles in a range of sizes from 50 to 500 nm. The level of targeting and uptake is directly proportional to the amount of targeting agent attached to the particles. However, it should be kept in mind that the targeting effect is greatly reduced in the presence of excess free lectin, or specific sugar.

Preclinical studies have suggested that paclitaxel is not significantly absorbed after oral administration due to poor absorption or extensive presystemic hepatic metabolism. Walle and Walle (1998) investigated the transepithelial flux of paclitaxel using Caco-2 cell line. The flux from the basolateral to the apical side was 4-10 times greater than that from the apical to the basolateral side. These data concluded that rapid passive diffusion of paclitaxel through the intestinal epithelium is partially counteracted by the action of an outwardly directed efflux pump, presumably P-glycoprotein, P-gp. It was confirmed that the expression of P-gp exists on the apical side of the human intestinal cells (Hunter et al., 1993) and that paclitaxel is a substrate of this transporter (Gupta, 1995; Horwitz et al., 1986). The ability of verapamil, a well-known P-glycoprotein antagonist (Racker et al., 1986), to reduce this active transport is additional evidence for the involvement of this mechanism. No metabolism of paclitaxel was observed in the Caco-2 cells.

2.3.5.2. Cellular uptake of polymeric nanoparticles

Biodegradable micro- (MP) and nanoparticles (NP) are being widely investigated as systems to deliver poorly absorbed drugs including peptides, proteins and vaccines to mucosal surfaces (Couvreur and Puisieux, 1993). The specific requirements for the system to deliver the drugs effectively are high uptake by malignant cells, low affinity to normal tissues and satisfactory intracellular drug release kinetics to achieve therapeutic level.

The polymeric micro- and nanoparticles are of particular interest for oral administration as they provide protection of fragile molecules against enzymatic and hydrolytic degradation in the gastrointestinal tract (Fattal et al., 2002) and they can naturally be taken up by enterocytes (Damge et al., 2000; Florence, 1997) and/or the lymphoid tissues in Peyer's patches (PPs) (Tice, 1990; Jani et al., 1990). Although MPs and NPs have been found to be poorly absorbed by the intestine with only a small percentage of particles appearing in lymph nodes, blood, liver and spleen (Nefzger et al., 1984), drug-loading of particles has been reported to enhance or prolong the peroral bioavailability of a number of drugs. Uptake of biodegradable NPs and MPs by the gut associated lymphatic tissue after oral administration has received a lot of interest because of the possibility of using these carriers to deliver by the oral route, antigens or DNA vaccines.

The uptake is supposed to be dependent on particle's physicochemical properties such as the size, zeta potential and hydrophobicity. Eldridge et al. (1990) have illustrated the potential of microspheres as a controlled-release delivery system which targets the Peyer's patches when orally administered. Microspheres made of numerous biodegradable and non-biodegradable polymers were seen to be absorbed by the

microfold cells of the Peyer's patches and passed to the underlying leukocytes. Size was found to be a determinant controlling absorption. They reported that orally administered 1-10 μm microspheres composed of poly (D,L-lactide-co-glycolide) (PLGA) were specifically taken up into the Peyer's patch lymphoid tissue of the gut in mice, and those greater than or equal to 5 μm remained for up to 35 days. The major determinant in the absorption of microspheres <10 μm is their effective hydrophobicity.

Desai and co-workers (1997) revealed the GI uptake of particles were dependent on diameter and concentration of particles, and incubation time and temperature. The 99.4% (w/w) association of fluorescence with particles confirmed fluorescent intensity measured in the cells was mainly due to the uptake of fluorescent particles. The small particles of 100 nm had uptake significantly (2.5 fold) greater than larger size particles (1 μm) and mostly localized in the endosomes as evident from the confocal microscopy studies. They concluded that smaller size particles with enhanced intestinal epithelium uptake could be ideal carriers for intestinal administration of sustained release vaccines.

Desai et al. (1997) and McClean et al. (1998) observed a distinct uptake of large particles from 1 μm (15%) up to 10 μm (6%) without any loss of monolayer barrier function suggesting the mechanism of uptake in Caco-2 cell is size dependent. Kofler et al. (1997) also revealed size and surface characteristic-dependent uptake of particles into Peyer's patches indicating small particle size, rapid antigen release and high antigen content provide optimal tools to deliver orally applied antigens.

Suh and colleagues (1998) have investigated the formulation and the effect of paclitaxel-loaded PEO-PLGA nanoparticles (~ 150 nm) on the vascular smooth

muscle cells (VSMC). Paclitaxel-loaded particles exhibited comparable anti-proliferative effect to the free paclitaxel and the cell viability was reversible. The sustained drug release profile and cellular internalization suggest that paclitaxel-loaded nanoparticles may potentially be used as an endocytizable, local sustained release drug delivery system.

The studies have revealed the controlling factors of cellular uptake as size, nature of the polymer, zeta potential, coating and presence of nutrients (Delie, 1998). Due to numerous discrepancies in the literature, the best design of drug carrier system cannot be determined.

2.3.5.3. Mechanisms of uptake of particles in the gastrointestinal tract

The particle absorption pathway and efficiency in the GI tract highly depend on the size of particles administered (Desai et al., 1996) and particles larger than 10 μm were not taken up. Investigations of intestinal particle uptake can be performed using different models under in vivo (Desai et al., 1996), in situ (Pappo and Ermak, 1989), ex vivo (Scherer et al., 1993) or in vitro conditions (Delie, 1998; Desai et al., 1997; Norris and Sinko, 1997).

Consensus has not been reached on the mechanisms of particle uptake by intestinal epithelia. Most evidence suggests that the favoured site for uptake is the PP lympho-epithelial M cell (Lefevre et al., 1985; Eldridge et al., 1990; Jani et al., 1992). However, paracellular transport of particles has been favored by others (Volkheimer and Schulz, 1968; Aprahamian et al., 1987) while there is also evidence for particle endocytosis by intestinal enterocytes (Sanders and Ashworth, 1961; Kreuter et al.,

1989). It was recently reported that particles in the size range 40 to 120 nm were translocated both transcellularly and paracellularly (Mathiowitz et al., 1997).

2.3.5.3.1. Paracellular uptake

Volkheimer (1977) attributed the passage of particulates through the mucosa of the small intestine to persorption which involved "kneading" of the particle between intestinal epithelial cells. Significant paracellular transport of macromolecules and particles is an unlikely event since the spaces between tight junctions was reported to be <10Å. Paracellular permeability for peptides can be enhanced by polymers such as chitosan, poly (acrylate), or starch (Jung et al., 2000). However, these hypotheses were derived mostly from cell culture models and their relevance for the in vivo situation is unclear.

2.3.5.3.2. Endocytotic (Intracellular) uptake

Sanders and Ashworth (1961) observed 220 nm polystyrene particles in rat intestinal epithelial cells one-hour post intra-gastric administration, which suggested possible particle absorption by intestinal enterocytes through endocytosis. The particles would be then included in cytoplasmic vesicles and discharged in the serosal spaces to gain access to the mesenteric lymph or blood. This is supported by Florence and colleagues (1995).

Endocytotic processes are characterized by pinching of membrane vesicles from the plasma membrane, followed by an internalization of the engulfed extracellular materials (Jung et al., 2000). Of particular interests are transcytotic processes at mucosal surfaces by which macromolecules or particles, internalized at the apical

plasma membrane of the epithelial cells, are transported to the contralateral plasma membrane and released to the basolateral compartment (Fig. 2.3, II-III). Receptor-mediated endocytosis (RME) requires receptors at the apical cell membrane and actively transports particles of sizes up to 500 nm. Adsorptive endocytosis does not require receptors but depends on size and surface properties of the adsorbed material (Jung et al., 2000). Thus, macromolecules and colloidal carriers (submicron) may have potential for oral delivery via this mechanism although its efficiency remains to be demonstrated.

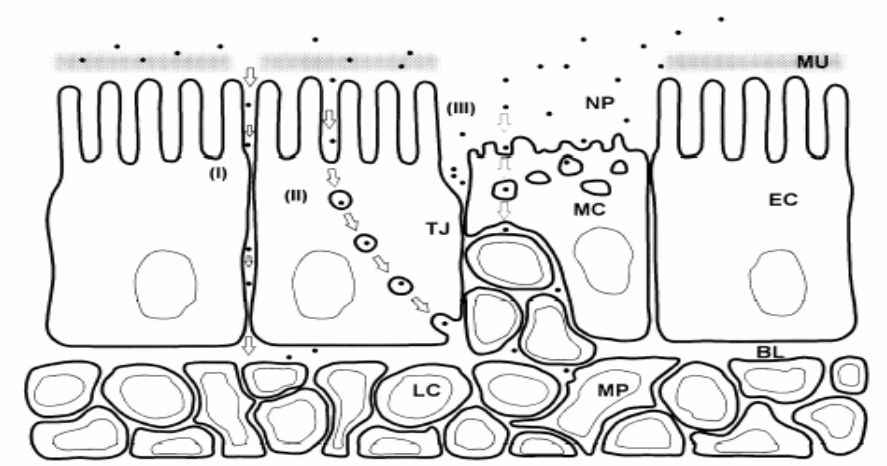


Figure 2. 3. Schematic drawing of mucus (MU) covered absorptive enterocytes (EC) and M cells (MC) in the small intestine. Lymphocytes (LC) and macrophages (MP) from underlying lymphoid tissue can pass the basal lamina (BL) and reach the epithelial cell layer which is sealed by tight junctions (TJ). Possible translocation routes for NP are (I) paracellular uptake, (II) endocytotic uptake by enterocytes and (III) M cells. (From Jung et al., 2000).

2.3.5.3.3. Lymphatic uptake

The absorption of particulates (≤10 μm) predominantly takes place at the intestinal lymphatic tissues (i.e. Peyer's patches) (Hillery et al., 1994, Lefevre et al., 1978). The epithelial cell layer overlying the Peyer's patches contains specialized M cells which are believed to be transcytotic (Pappo and Ermak, 1989). Particles in the intestinal

lumen localize to M cell apical surfaces and can be internalized by M cells through transcytosis.

2.3.5.4. Cytotoxicity study of drug-loaded polymeric nanoparticles

The therapeutic effects of the drug-loaded polymeric nanoparticles can also be demonstrated by determining the viability of the cancer cells under equivalent doses over the same period of treatment as with the current clinical formulation. The viability of cells after treatment with the drug formulations can be evaluated by the MTT assay. This assay is based on the cellular reductive capacity of living cells to metabolize the yellow tetrazolium salt, 3-(4, 5-dimethylthizaol-2-yl)-2, 5, 5-diphenyl tetrazolium bromide (MTT), to a chromophore, formazan product, whose absorbance can be determined by spectrophotometric measurement. The number of viable cells is directly related to the measured absorbance which represents the amount of formazan formed by living cells.

The cytotoxic activity of paclitaxel or loaded in PLGA nanoparticles was compared with formulation in Cremophor EL by assessing cell viability by the MTT assay using NCI-H69 cell line (Fonseca et al., 2002). A much higher cytotoxic effect could be observed for nanoparticle formulation after a long incubation time (168 hrs) under the same paclitaxel concentration. Another study reported (Suh et al., 1998) that paclitaxel incorporated PEO-PLGA nanoparticles of 150nm showed comparable anti-proliferative effects on vascular smooth muscle cells as free paclitaxel.

2.3.6. *In vivo* evaluation by animal models

In vivo animal models are applied to investigate the effects and efficacy of specific delivery system. In vivo studies give essential information about absorption of particles and it is the only means to determine the actual rate of uptake of ingested particles. However, inter-species and inter-animal differences will limit extrapolation to other models and prevent study comparison. Rate of uptake may also change according to the age of the animal. Kofler et al. (1997) revealed size and surface characteristic-dependent uptake of microspheres using BALB/c mice; efficient microsphere translocation into Peyer's patches for 0.8 μm microspheres but poor for 2 μm and surface-modified microspheres.

CHAPTER 3

FORMULATION AND CHARACTERIZATION OF PLGA NANOPARTICLES FOR ORAL PACLITAXEL ADMINISTRATION

3.1. Introduction

3.1.1. Significance of drug delivery system

Controlled release of drugs, proteins, and other bioactive agents can be achieved by incorporating them in dissolved or dispersed form in polymers (Baker and Lonsdale, 1974). The controlled drug delivery systems draw increased attention due to its enhanced efficacy of existing potent drugs at lesser expenses and fewer dosing schedule. Controlled delivery system maintains the drug level in the blood between the maximum and minimum therapeutic levels at a minimum dosage for an extended period of time (Karsa, 1996; Dunn, 1991). Conventional delivery system provides fluctuated drug level in the blood, either exceeding the maximum or falling below the minimum therapeutic level, resulting in toxic side effects or inefficacy. Most anticancer drugs have limitations in clinical administration due to their poor solubility and other unfavorable properties. Thus, biodegradable polymeric controlled delivery systems may bring the higher drug efficacy and better applications for the potent anti-cancer drugs.

Nanotechnology permits the delivery of drugs that are highly water-insoluble or unstable in the biological environment. It is expected that novel drug delivery systems can make a significant contribution to global pharmaceutical sales. This is illustrated

by the fact that approximately 13% of the current global pharmaceutical market is accounted for by sales of products incorporating a drug delivery system. The demand for drug delivery systems in the United States alone is expected to grow nearly 9% annually to more than US$82 billion by 2007 (Sahoo and Labhasetwar, 2003).

Many therapeutic agents have not been successful because of their limited ability to reach to the target tissue. Although opportunities to develop nanotechnology-based efficient drug delivery systems extend into all therapeutic classes of pharmaceuticals, the faster growth opportunities are expected in developing delivery systems for anticancer agents, hormones and vaccines because of safety and efficacy shortcomings in their conventional administration modalities.

3.1.2. Need of efficient drug delivery system for novel anticancer drug, paclitaxel

Paclitaxel, the first of a new class of microtubule stabilizing agents, has been hailed by National Cancer Institute (NCI) as the significant advance in chemotherapy of the past few decades. Paclitaxel was not a chance discovery but the outcome of the investigation over 12,000 natural compounds for anticancer activity (Appendino, 1993). Paclitaxel is a promising anti-tumor agent with poor water solubility. Hence, the current formulation of intravenous (i.v.) administration is in a non-aqueous vehicle containing Cremophor EL which may cause various and serious side-effects and precipitation on aqueous dilution. The extensive clinical use of this drug is somewhat delayed due to the lack of appropriate delivery vehicles.

Since best medical effects are yet to be achieved, it becomes necessary to come up with improved alternate formulations of paclitaxel for a more effective clinical

administration that eliminates premedication and the toxicity of the adjuvant CrEL, reduces side-effects to the least and also improves drug efficacy. The desired formulation should be one that does not consume toxic adjuvant, is capable of releasing paclitaxel over an extended period of time and stabilizing the compound during long term storage of the formulation but yet economically feasible to be produced on a large scale. Various approaches employed to date include cosolvents, emulsions, micelles, liposomes, microspheres nanoparticles, cyclodextrins, pastes, and implants.

3.1.3. Preparation of nanoparticles by emulsification-solvent evaporation method

Emulsification-solvent evaporation is one of the oldest and most widely used methods of particle preparation and the most suitable process for encapsulation of hydrophobic drugs. The solubility of the drug and polymer in the organic solvent is a major factor in determining the morphology of particles produced by the solvent evaporation process and the final state of the polymer itself (crystalline or amorphous). The size of the particles achieved substantially depends on the speed and rate of the agitation when the oil phase is added to the aqueous phase to form emulsion.

The solvent evaporation is conceptually simple, but a large number of process variables exist which can profoundly affect the nature of the product obtained. Some parameters affecting solvent evaporation method are polymer molecular weight and concentration, polymer crystallization, type of drug and method of incorporation, organic solvent used, type of surfactant in aqueous phase, organic solvent/aqueous phase ratio, evaporation temperature and rate of stirring.

Semicrystalline polymers often give porous structures with spherulites on the surface of the nanoparticles whereas uniform, pore-free nanoparticles are most readily obtained with amorphous polymers. One requirement of this process is that the active agent (i.e., the drug) partition favorably into the oil phase. This partitioning is favored by active agents that are insoluble or sparsely soluble in water. Such agents may be insoluble in the organic solvent. In the latter case, the nanoparticles contain a dispersion of crystals embedded in a polymer matrix. The crystals should be the same ones in the polymer solvent solution, assuming that there is no partial solubilization and/or recrystallization. If the active agent dissolves completely in the organic solvent phase, it may or may not form crystalline domain in the polymer matrix that makes up the nanoparticles.

Preventing migration of active agent into the aqueous phase from the solvent phase is often a problem. Crystalline active agents completely soluble in the solvent phase tend to form free crystals in the aqueous phase or on the surface of the microspheres. This effect can be minimized by removing the emulsifier from the system before solvent evaporation is complete.

The physicochemical properties of progesterone-loaded poly (L-lactide) microspheres prepared by the solvent evaporation method were reported by Izumikawa et al. (1991). Solvent removal at atmospheric pressure yielded microspheres of crystalline polymer matrices, whereas faster solvent removal under a reduced pressure gave microspheres of amorphous polymer matrices. The crystallinity of the polymer matrices was closely correlated with the morphology and physical properties of microspheres, and affected the drug release rate. The microspheres of crystalline polymer matrices had rough surfaces with large surface areas, and exhibited a rapid

drug release, whereas the amorphous microspheres provided a slower drug release. The results of X-ray diffraction (XRD), differential scanning calorimetry (DSC), and Fourier-transform infrared (FTIR) spectroscopy suggested that progesterone formed a molecular dispersion in the amorphous polymer matrices.

3.1.3.1. Selection of solvent

Ethyl acetate and methylene chloride are solvents of first choice due to their relatively lower toxicity. Methylene chloride is a preferred solvent due to its high volatility and its capacity for dissolving a broad range of polymers. It is used for extraction in the food and pharmaceutical industries and also in the extraction of fats and paraffin.

The use of methylene chloride may impose a problem in obtaining product approval by regulatory agencies. As evidenced by Lupron Depo™, the most successful microsphere product being used in human, a small amount of methylene chloride remaining in a microsphere product is acceptable by the USFDA, but only if the product's therapeutic benefits clearly outweigh a safety concern over the residual solvent. Effect of additives or emulsifiers on the drug release kinetics from biodegradable matrices is an important determination in designing a drug delivery system.

3.1.3.2. Selection of emulsifier

The emulsifier decreases the extent of droplet coalescence and coagulation and stabilizes the emulsion system. The emulsifiers have influential effects on particle size and size distribution, morphological properties and release profiles of the nanoparticles (Mu and Feng, 2002). In solvent evaporation method, hydrophilic

polymeric colloids and anionic or nonionic surfactants such as poly (vinyl alcohol), gelatin and sodium dodecyl sulfate are generally applied as emulsifiers. A suitable emulsifier or surfactant is required to produce a stable emulsion, a result achieved by lowering the surface tension, γ (usually from 40 to 5mN/m^{-1}). Moreover, for pharmaceutical applications, emulsifier must be acceptable for therapeutic use (Wade and Weller, 1994).

3.1.3.2.1. Poly (vinyl alcohol) (PVA)

Water soluble synthetic polymer, PVA, has excellent film forming, emulsifying and adhesive properties which make it a versatile polymer for many applications. PVA (Fig. 3.1) is one of the most frequently used emulsifiers due to its high viscosity and strong adsorption around the emulsion drops. However, excess PVA may not easily be washed out of the nanoparticles.

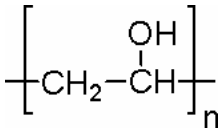


Figure 3. 1. Structure of poly (vinyl alcohol)

3.1.3.2.2. Poly (acrylic cid) (PAA)

Another water soluble polymer PAA (Fig. 3.2) has excellent water-holding capability and can be used in various applications in diverse fields including commercial and medical uses such as super absorbents, textile and drug delivery coatings. The bioadhesive properties of PAA which allow them to readily attach and conform to

accessible sites of the body found its application in producing bioadhesive polymer gel as components of medical sensors and electrically modulated drug delivery devices.

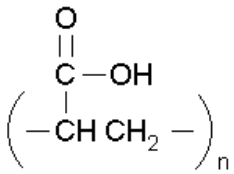


Figure 3. 2. Structure of PAA

3.1.3.2.3. Vitamin E-TPGS (TPGS)

Vitamin E-TPGS (D-α-topopheryl polyethylene glycol 1000 succinate) is a water-soluble derivative of natural vitamin E. It is formed by the esterification process between vitamin E succinate and polyethylene glycol 1000. It can be seen from the chemical structure (Fig. 3.3) that it has amphiphilic properties with the long alkyl tail contributing to its lipophilicity and the tocopherol succinate polar group contributing to its hydrophilicity. Therefore, it is a good candidate for emulsifying a wide range of water-oil immiscible systems. TPGS could be absorbed intact readily in the gastrointestinal tracts, and could inhibit P-glycoprotein in the intestine to enhance the cytotoxicity of anti-cancer drugs such as paclitaxel (Mu and Feng, 2002). TPGS finds its applications in drug delivery systems such as protecting the drug from absorption process and enhancing the bioavailability of poorly absorbed drug.

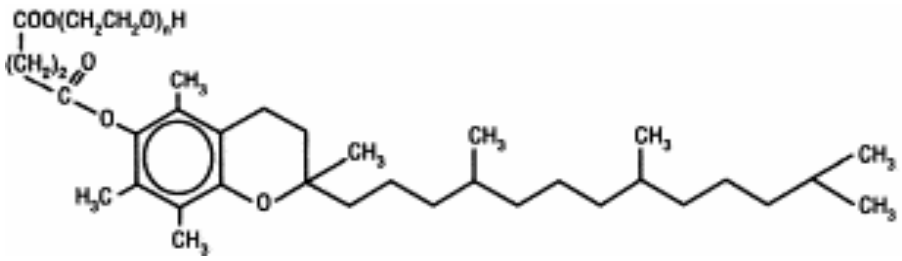


Figure 3. 3. Structure of vitamin E-TPGS

3.1.3.2.4. Phospholipid (DPPC)

Phospholipids have been widely used as natural emulsifiers and other purposes in industry although their application as emulsifier in drug delivery formulation has been rarely reported. Phospholipids are used as emulsifiers in the products such as animal feeds, baking products and mixes, chocolate, cosmetics and soaps, dyes, insecticides, paints and plastics. The use of 1,2-dipalmitoylphosphatidylcholine (DPPC, Fig 3.4) as an additive in drug delivery formulation was reported to improve the performance of the PLGA microspheres in blood flow (Garti, 1999), enhance the pulmonary absorption of peptides and proteins (Zhen et al., 1995), reduce phagocytic uptake of the microparticles (Evora et al., 1998), and impart better emulsifying effects and higher encapsulation of drug in the nanoparticles (Feng and Huang, 2001). Phospholipids exhibit high potential of application in drug delivery formulations.

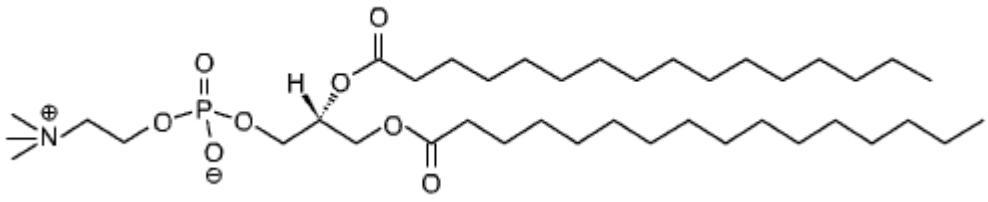


Figure 3. 4. Structure of DPPC

3.1.3.2.5. Monoolein

Although amphiphilic monoolein (Fig. 3.5) is commonly used as an emulsifying agent and as a food additive since the 1950s, its potential applications in the pharmaceutical industry has been increasingly discussed recently. Monoolein is a nontoxic, biodegradable, and biocompatible material classified as GRAS (generally recognized as safe), and it is included in the FDA *Inactive Ingredients Guide* and in non-parenteral medicines licensed in the United Kingdom (Ganem-Quintanar et al., 2000). Its biodegradability comes from the fact that monoolein is subject to lipolysis due to diverse kinds of esterase activity in different tissues (Longer et al., 1996). In 1984, monoolein was first proposed as a biocompatible encapsulating and controlled-release material. Since then, there have been many diverse applications, and new uses proposed.

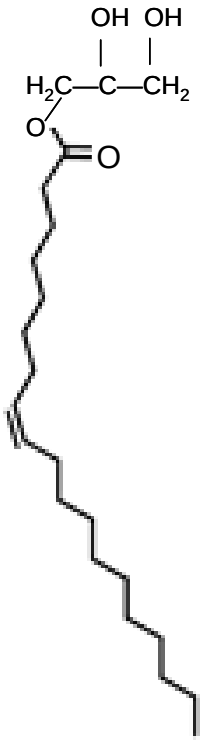


Figure 3. 5. Structure of monoolein

Ganem-Quintanar et al. (1996) classified the applications of monoolein in the pharmaceutical field as: (1) emulsifier (nonionic surfactant), (2) solubilizer, (3) absorption enhancer, (4) oral drug delivery system, (5) parenteral drug delivery system, (6) vaginal drug delivery system, (7) periodontal drug delivery system, (8) colloidal carrier system, (9) storage system for the protection of macromolecules susceptible to degradation, (10) bioadhesive, and (11) others, such as a membrane and polymorphic lipid model for the determination of lipid bilayer/water partition coefficient and electrochemical biosensors.

3.1.3.2.6. Montmorillonite (MMT)

Increased use of chemically inert clay for cosmetic and medical applications was found in the recent years. The two most industrially important clay minerals of smectite group are sodium montmorillonite and calcium montmorillonite. Montmorillonite (MMT) belongs to the structural family of the 2:1 phyllosilicates. MMT is the most commonly used layered silicates and its structure is shown in Fig. 3.6. Their special characteristics such as leaflet structure, high plastic viscosity, antacid and antidiarrhea properties, make them ideal for use as pharmaceutical recipients, or as active ingredients, or as colloidal stabilizers in the emulsion.

Certain pharmaceutical materials including clay exhibit mucoadhesive properties. When formulated in certain proportions in aqueous colloidal dispersions with drug and in the form of a flowable liquid, these are able to interact with glycoprotein transforming into viscous gel to become effective mucoadhesive systems. Therefore, MMT is a good candidate to be incorporated in the oral drug delivery systems.

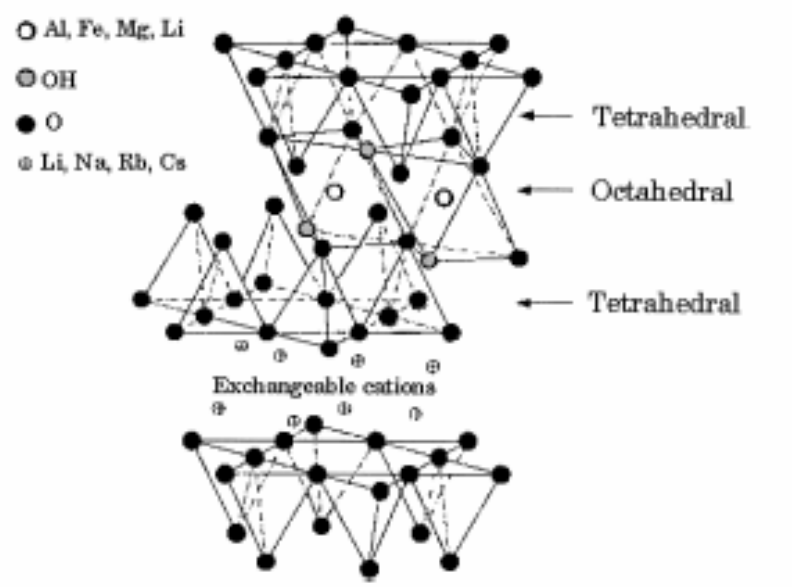


Figure 3. 6. Structure of 2:1 Phyllosilicates

3.2. Experimental methods

3.2.1. Materials

Paclitaxel of purity 99.8% was purchased from Dabur India Ltd. (India). Poly (D, L-lactic-co-glycolic acid) (PLGA) with L:G molar ratio of 50:50 and MW of 40,000–75,000, polyvinyl alcohol (PVA) with MW of 30,000–70,000, fluorescence marker (coumarin-6), 1,2-dipalmitoylphosphatidylcholine (DPPC), phosphate buffered saline (PBS), MEM medium, penicillin-streptomycin solution, Trypsin-EDTA solution, Triton® X-100 and Hank's balanced salt solution (HBSS) were purchased from Sigma (St. Louis, MO, USA). Vitamin E d-α-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS or simply TPGS) was obtained from Eastman (TN, USA). Distilled monooleate was from Grindsted products (Brabrand, Denmark). Montmorillonite K10 was from Fluka (Buchs, Switzerland). Dichloromethane (DCM, analytical grade) was from Merck (Darmstadt, Germany) and acetonitrile (HPLC grade) was from Fisher

Scientific (NJ, USA). Fluorescent polystyrene nanoparticles were purchased from Duke Scientific (CA, USA). Fetal bovine serum (FBS) was received from Gibco (Life Technologies, AG, Switzerland). Ultrapure water (Millipore, Bedford, MA, USA) was used throughout the experiment.

3.2.2. Preparation of nanoparticles

Nanoparticles, placebo or loaded with paclitaxel or fluorescent marker were prepared by a modified solvent extraction/evaporation method (single emulsion). In brief, an oil phase solution of dichloromethane (DCM) containing poly (DL-lactide-co-glycolide) (PLGA, 50:50) was slowly poured into an aqueous solution containing emulsifiers such as PVA, vitamin E TPGS, etc. The polymer solution also contained paclitaxel or 0.05% (w/v) coumarin-6 as fluorescent marker in preparation of fluorescent nanoparticles. This mixture was homogenized for 30 sec by vortexing followed by sonication (Microson XL2000, Misonix Inc, NY) set at an energy output for 90 sec in pulse mode to produce the oil-in-water (o/w) emulsion. The o/w emulsion was stirred over a magnetic stirrer plate overnight at room temperature to evaporate dichloromethane and harden the nanoparticles formed. The nanoparticles were collected by centrifugation (5810R, Eppendorf, 10,000 rpm, 15min, 18ºC) and washed with Millipore water for 3 times to remove excessive emulsifier and fluorescent marker. The nanoparticle suspension was then freeze-dried (Christ, Alpha-2, Martin Christ, Germany) to obtain fine powder of nanoparticles, which was kept in a vacuum desiccator. Some emulsifiers were applied to compare the acquired physicochemical properties of nanoparticles and choose the most potential formulation for delivery of anti-cancer dugs. Formulation optimization was pursued to obtain nanoparticles of desired physicochemical properties.

3.2.3. Characterization of nanoparticles

3.2.3.1. Size and size distribution

Nanoparticle size and size distribution were determined by laser light scattering with particle size analyzer (90 Plus, Brookhaven Inst, Huntsville, NY, USA) at a fixed angle of 90° at 25°C. In brief, the dried nanoparticles were suspended in filtered deionized water and sonicated to prevent particle aggregation and to form uniform dispersion of nanoparticles. The size distribution was given by the polydispersity index. The lower the value is, the narrower the size distribution or the more uniform of the nanoparticle sample. The data reported represent the average of 5 measurements.

3.2.3.2. Surface Morphology

Morphology of the formulated nanoparticles was observed by scanning electron microscopy (SEM, Jeol JSM 5600LV) at 10-15kV. SEM sample preparation requires an ion coating with platinum by a sputter coater (JFC-1300, Jeol, Tokyo) for 40 seconds in a vacuum at a current intensity of 40 mA after preparing the sample on metallic studs with double-sided conductive tape.

3.2.3.3. Surface charge

Zeta potential is an indicator of surface charge, which determines particle stability in dispersion. Zeta potential of nanoparticles was determined by a zeta potential analyzer (Zeta Plus, Brookhaven Instruments, Huntsville, NY) by dipping a palladium

electrode in the sonicated particle suspension. The mean value of 10 readings was reported.

3.2.3.4. Yield of nanoparticles

The overall yield of particles can be calculated as follows:

$$Yield\ (\%) = \frac{amount\ of\ particles\ obtained}{initial\ amount\ of\ polymer\ (and\ drug)} \times 100 \qquad (3.1)$$

3.2.3.5. Drug loading

The drug loading or drug content is expressed as:

$$Drug\ loading = \frac{amount\ of\ drug\ encapsulated\ in\ particles}{amount\ of\ particles} \times 100 \qquad (3.2)$$

3.2.3.6. Encapsulation efficiency

Encapsulation efficiency is defined as:

$$Encapsulation\ efficiency\ (EE,\ \%) = \frac{drug\ loaded\ in\ particles}{initial\ amount\ of\ drug\ added} \times 100 \qquad (3.3)$$

To determine encapsulation efficiency, 3 mg of particles were dissolved in 1 ml of DCM followed by the addition of 3 ml of acetonitrile:water (50:50, v/v) solution to extract the drug to be analyzed by high performance liquid chromatography (HPLC). DCM was evaporated at room temperature under nitrogen stream until clear solution

was obtained. To determine the recovery efficiency of paclitaxel from the extraction process, known weights of paclitaxel from 100 to 1000 μg and 3.0 mg of placebo particles were subjected to the same extraction procedure. The recovery was 91.99 % which implies that 92 % of paclitaxel was detected and thus all the data obtained were corrected before calculating the encapsulation efficiency.

3.2.3.7. X-ray diffraction (XRD) analysis

X-ray powder diffration analyses were performed on pure paclitaxel, pure emulsifiers (PVA, TPGS and DPPC) and paclitaxel-loaded PLGA nanoparticles incorporating them, in order to find out whether paclitaxel was present in the nanoparticles in a crystalline or an amorphous state. A Shimadzu X-ray Diffractometer (XRD-6000) was used and samples were exposed to monochromatic CuKα radiation (40 kV x 30 mA, λ = 1.5406 Å). The diffraction pattern was determined in the area of 5° <2θ< 45°, and the step size was 0.02° and the step time was 0.6 s.

3.2.4. *In vitro* paclitaxel release studies

Nanoparticles were dispersed in the simulated physiological fluid, phosphate buffered saline (PBS) at pH 7.4. The buffer solution was kept in an orbital shaker at constant gentle shaking of 110 rpm and physiological temperature of 37°C. At predetermined time intervals, the suspensions were centrifuged at 11,000 rpm and 37°C for 18 min. The precipitated particles were re-suspended in fresh buffer and placed back in the shaker. The supernatant was kept for HPLC analysis after the drug was extracted with DCM followed by mobile phase (acetonitrile:water, 50;50, v/v). For HPLC analysis, a reverse phase Inertsil® ODS-3 column (150x4.6 mm i.d., pore size 5 μm,

GL Science, Tokyo, Japan) was used and the mobile phase was delivered at a rate of 1 ml/min by a pump (Perkin Elmer-S200). 50 µl of sample was injected by an auto-sampler (Perkin Elmer-ISS200) and the column effluent was detected at 227 nm with a UV detector. The calibration curve was prepared for the quantification of drug in the particles and it was linear over the range of 50-10,000 ng/ml with a coefficient of $r^2 = 0.9999$.

Due to inefficient extraction process of the highly hydrophobic paclitaxel, the extraction factor had to be determined as follows: a known amount of paclitaxel was subjected to the extraction procedures in exactly the same manner as mentioned above and the actual amount of paclitaxel in the extracted samples was measured. It was found that only 43.56 % of the original paclitaxel was detected after the extraction process. Therefore, all the data obtained from in vitro release studies should be corrected accordingly.

3.2.5. Degradation studies of nanoparticles

To observe the degradation of particles *in vitro* and to find a relation between degradation of polymer and drug release rate, 3 mg of particles were dispersed in 2 ml of PBS buffer and incubated in a shaker bath maintained at 37°C and 110 rpm to simulate the physiological conditions. At specified time intervals, particles were collected by centrifugation followed by freeze-drying. The change in morphology was observed by SEM and the change in molecular weight was determined by gel permeation chromatography (GPC) as described below.

The freeze-dried nanoparticles (0.9–1.2 mg) were dissolved in 1.5 ml of stabilized HPLC-grade tetrahydrofuran (THF) and the solutions were filtered through 0.45 µm

syringe filters. The filtered solutions of 20 μl were manually injected and analyzed using THF as the mobile phase with a flow rate of 1ml/min. HP1100 series HPLC system equipped with HP1047A RI detector and Plgel Mixed-C column (Agilent, 300x7.5mm, 5μm) was employed. The data collection and analysis were done using the software provided by the manufacturer. Molecular masses were calculated from a calibration curve using a series of polystyrene standards (Polymer Labs, MA) with molecular mass range of 1270-5000000.

3.3. Results and Discussion

3.3.1. Formulation and characterization of nanoparticles

Nanoparticles with/without paclitaxel were successfully fabricated by oil-in-water emulsification/solvent evaporation process using both natural and synthetic emulsifiers such as PVA, PAA, TPGS, DPPC, monoolein, and montmorillonite. By varying the appropriate preparation parameters, different sizes, stability and encapsulation efficiency of particles may be obtained. Most importantly, the release profile of this drug delivery system can thus be modified. The particles prepared were freeze-dried to enhance stability and storage. The presence of an appropriate amount of suitable surfactant can increase the drug's solubility in the continuous phase and thus can affect encapsulation efficiency and release profile (Giunchedi et al., 1998, Wang et al., 2001).

PVA was conventionally used in emulsification method and thus PVA emulsified nanoparticles were fabricated as controls. Vitamin E TPGS was chosen as surface modification material of nanoparticles and emulsifier since it was claimed to

solubilize lipophilic compounds (Wang et al, 2001, 2002; Sokol et al., 1991; Boudreaux et al., 1993), and enhance EE of drug (Mu and Feng, 2002). Moreover, TPGS could be absorbed intact readily in the GI tract and could inhibit P-gp in the intestine to enhance absorption and oral bioavailability (Dintaman and Silverman, 1999; Sokol et al., 1991; Boudreaux et al., 1993). A natural phospholipid, DPPC, was employed because it was suggested to provide better coating and higher EE of drug (Feng and Huang, 2001), improve the performance of produced nanoparticles in the blood flow (Garti, 1999), enhance the pulmonary absorption of peptides and proteins (Zhen et al., 1995), and reduce phagocytic uptake of the nanoparticles (Evora et al., 1998).

Particles made of bioadhesive or mucoadhesive materials which can adhere to the mucus layer in the intestine have been widely studied to improve particle delivery efficiency. Mucoadhesive polymers can be used to formulate particulates which bind to intestinal mucus layer and thus slow down the intestinal transit. Therefore, they result in a prolonged intestinal residence time for orally administered particles and showed increased particle absorption efficiency in the animals studied.

Bioadhesive properties of PAA had attracted to give it a try in nanoparticle formulation for paclitaxel delivery. Amphiphilic monoolein was used because it is bioadhesive (Engström et al., 1995), non-toxic, able to incorporate both hydrophilic and hydrophobic drugs. Besides, it can act as emulsifier, solubilizer, and absorption enhancer. Montmorillonite was applied due to its properties (Tsugita et al., 1983) of colloidal stabilizer in oil-in-water emulsions, mucoadhesive material.

The results showed that TPGS and DPPC were very effective emulsifiers, better choices among the emulsifiers tested, at improving the emulsification process for

microencapsulation, in agreement with the recognized reported marvelous functions of phospholipids and vitamin E TPGS as novel emulsifiers in nanoparticle formulation of paclitaxel and other anticancer drugs (Feng and Huang, 2001; Mu and Feng, 2002). In addition to the right choice of the emulsifier for particle formulation, the amount of emulsifier added plays a fundamental role in determining the physicochemical properties of the formulated nanoparticles. Since the role of emulsifier is to stay on the interface of the nanoparticles to separate the oil and the water phase to stabilize the emulsion; and the smaller particles possess the larger total surface area for a given amount of their mass, too little emulsifier would not be enough to cover the interface while too much emulsifier would cause aggregation of the formed nanoparticles. Moreover, some drug molecules may bind to the excessive emulsifier molecules and thus cause the loss of drugs in the washing process. In general, small amount of emulsifier would result in large particles while large amount of emulsifier would result in reduced drug encapsulation efficiency. Thus, there is an optimal amount of the emulsifier which would result in the desired properties of nanoparticles. In preparation of nanoparticles by the solvent extraction/evaporation method, the concentration needed for the traditional emulsifier PVA was normally at least 1% (w/v) (Mu and Feng, 2002; Jefferey et al., 1991) while the required amount of TPGS is only 0.015% (w/v) and DPPC is 0.3% (w/v), an amount 67- and 7-times less than the PVA but with the same emulsifying effects. This implies that TPGS and DPPC have much higher emulsifying efficiency as much lower amount of TPGS and DPPC was needed, comparing to PVA, to have the comparable properties of nanoparticles.

3.3.2. Size and size distribution, yield, encapsulation efficiency and drug loading

The mean size and size distribution, yield, encapsulation efficiency and drug loading of the paclitaxel-loaded particles were summarized in Table 3.1. The narrow size distribution with high yield and high encapsulation efficiency is desired to achieve more consistent sustained efficacy and an economically feasible system. The low yield of particles with monoolein as emulsifier could be due to loss of smaller particles in washing process since its high polydispersity indicated wide size distribution of particles. In general, the size became bigger as it was expected when the drug was encapsulated in the particles. Paclitaxel encapsulation efficiency was higher than 55%, varying according to emulsifier type, except for MMT incorporated formulation.

Table 3. 1. Characteristics of Paclitaxel loaded PLGA 50:50 nanoparticles

Sample ID	Emulsifier (w/v)	Yield (%)	Size ± SD (nm)	Polydis-persity*	EE (%)
PVA	2% PVA	47.7	294 ± 4.8	0.110	92.8
PAAL	1% PAA (low Mw)	69.4	830 ± 50	0.152	81.0
PAAH	1% PAA (high Mw)	39.0	708 ± 13	0.005	72.8
TPGS	0.03% TPGS	44.9	375 ± 15	0.222	55.7
DPPC	0.3% DPPC	83.9	263 ± 3.9	0.046	61.8
Monoolein	1% monoolein	30.0	465 ± 7.0	0.280	61.7
MMT	1% montmorillonite	66.8	610 ± 13	0.225	13.9

* Polydispersity = the size distribution width

It is well known that the size of particles is highly dependent on the preparation method and conditions employed. Though it was generally believed that an increase in the percentage of the polymer affected the particle size, no significant effect on particle size was observed in the polymer concentration range from 1 to 5% (w/w). It

might be due to low polymer concentration range investigated. However, it was reported to have noticeable effect on size at a much higher polymer percentage of 25% w/w (Konan et al., 2001). It was postulated that the higher concentration of polymer in the preparation might have led to an increased frequency of collisions, resulting in fusion of semi-formed particles and producing an overall increase in the size of the particles.

The amount of emulsifier added can influence particle size since it can change the viscosity of the continuous phase and hence the formation of particles. If the emulsifier is not sufficiently added, the particle cannot be completely coated and separated from each other, which leads to aggregation and bigger sized particles. On the other hand, if the emulsifier is added in excess, the suspension is not well separated due to excess emulsifier in the solution, which again leads to the formation of bigger sized particles and a sheet of spread particles. Moreover, drug will interact with excess emulsifier and encapsulation efficiency will be decreased. Thus, it should be optimized to get well-separated and coated particles for better delivery of drug. From Table 3.2, it can be seen that 0.5% and 2% (w/v) PVA did not make much difference in size of the particles although the yield of the nanoparticles was a bit higher at 2%. When it was increased to 5% (w/v) PVA, the size of the particles became almost 3 times bigger than at 2% PVA. Thus, 2% was selected as the optimal amount of PVA that should be used in the formulation.

Table 3. 2. Effect of emulsifier amount on characteristics of PLGA 50:50 nanoparticles

Emulsifier (w/v)	Size ± SD (nm)	Polydispersity	Yield (%)
0.5% PVA	219.4 ± 10.5	0.144	65.8
2% PVA	212.5 ± 7.5	0.098	72.8
5% PVA	702.0 ± 56.6	0.425	70.2

When the volume of aqueous phase was increased, the mixing efficiency is decreased due to high volume of mixture, or high loading to the sonicator. The reduction in mixing efficiency probably produced an increase in the size of the emulsion droplets formed during the process, which would result in the formation of larger particles.

It was found, as expected, that the higher the sonication strength, the smaller the particles obtained. When the sonication was done at an output of 12W, the size of particles was about half of that of particles prepared at 6W while keeping all the other parameters the same. However, the mixing time did not give noticeable difference in size of particles when it was increased from 1 minute to 5 minutes. The nanoparticle preparation process parameters stated in Table 3.1 were chosen, from the formulation optimization, for further studies by characterization and in vitro evaluation of nanoparticles.

The results regarding the particle size, obtained from the two different techniques (90Plus and SEM), sometimes show large differences. Typically, the 90Plus particle sizer demonstrated mean volume diameters much higher than those given by SEM analyses (number size average). The difference in size determining technique and the effect of freeze-drying could also be responsible for this inconsistency. With this point in mind, the particle size was determined before the freeze-drying and it was found to be in agreement with the SEM analysis. Thus, it was assumed there might be some aggregations in the suspension caused by either freeze-drying or inadequate dispersion.

Numerous studies have shown the cryoprotective effect of excipients such as sugars to prevent particle aggregation during the freeze-drying process (Mehnert and Mader, 2001; Konan et al., 2001; Chasteigner et al., 1996). The effectiveness of

cryoprotective additive D-Trehalose (Sigma, St. Louis, MO) against the adverse effects of freeze-drying on the particle redispersibility was evaluated by varying the concentration and the addition step in the process since trehalose was claimed to be the best cryoprotectant (Mehnert and Mader, 2001; Konan et al., 2001). When D-Trehalose was included in the preparation process rather than in the suspension prior to freeze-drying, it was observed to produce well-separated and non-aggregated particles. The size determination showed the close results before and after freeze-drying. It seemed to be one option to prevent particle aggregation and different size analyzing results for future preparation procedures.

3.3.3. Morphology of nanoparticles

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) have been used to characterize the surface morphology of paclitaxel loaded PLGA nanoparticles fabricated with various emulsifiers. Figure 3.7 shows the SEM images of paclitaxel loaded PLGA nanoparticles formulated with (a) PVA, (b) vitamin E TPGS, (c) monoolein, (d) montmorillonite, (e) DPPC, and (f) PAA (low Mw) as emulsifier (Bar = 1μm). Under SEM examination, nanoparticles of all formulations exhibited spherical morphology and smooth surfaces, confirming results obtained by 90Plus size analysis. No significant difference in morphology can be observed between each sample. However, nanoparticles incorporated with monoolein and MMT showed a wider size distribution, and furthermore, particles did not properly form up to the preferred physicochemical properties. Therefore, nanoparticles incorporating PVA, TPGS and DPPC were selected for further detailed characterization and evaluation of their efficacy.

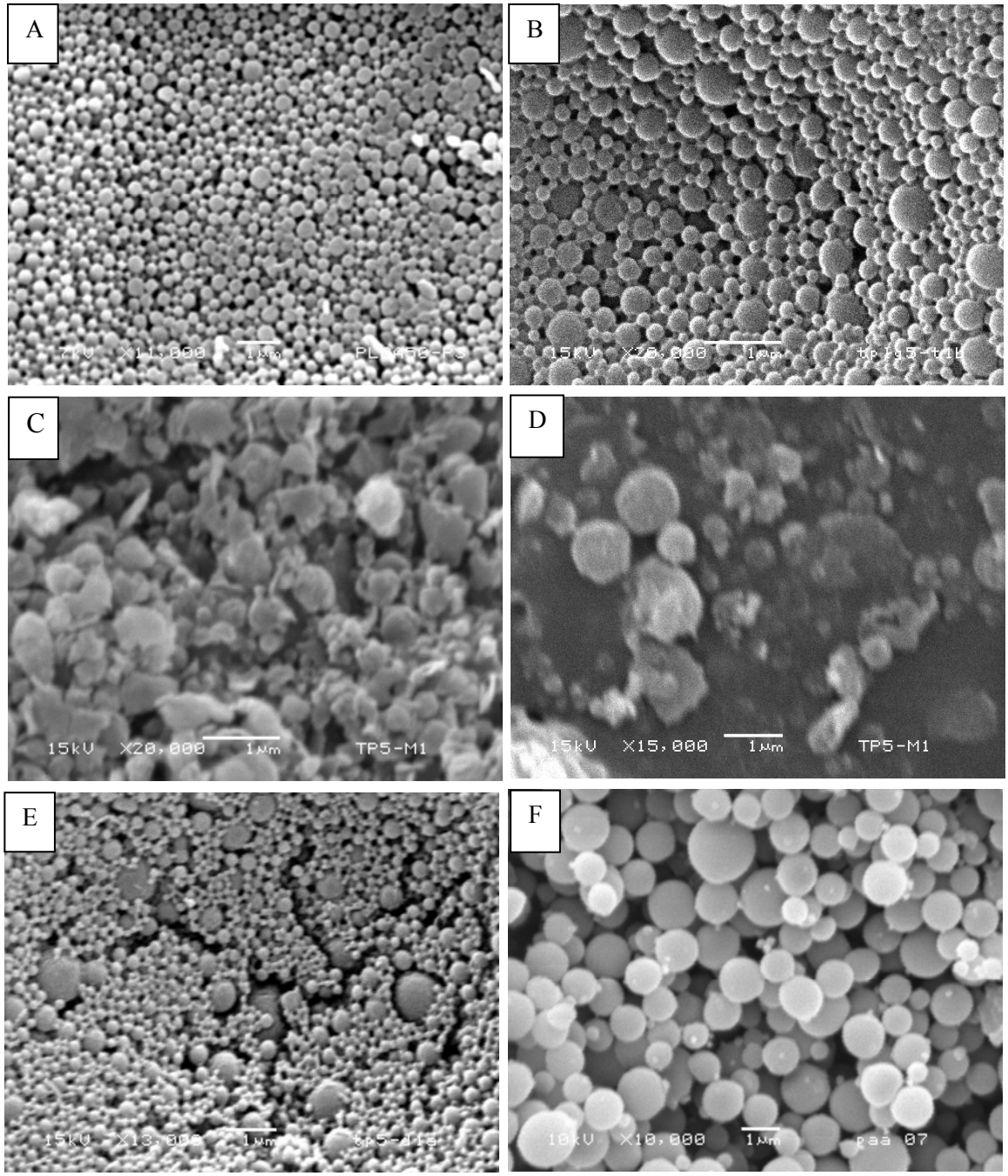


Figure 3. 7. SEM images of paclitaxel-loaded PLGA particles with emulsifier: A) PVA; B) vitamin E TPGS; C) monoolein; D) montmorillonite; E) DPPC; F) PAA (low Mw).

AFM technique has been widely applied to produce surface-dependent information in three dimensions on the nanometer scale. It is capable of resolving surface details down to the atomic level and can give morphological images in high resolution (Mu

and Feng, 2002). Thus, AFM is ideally suited for characterization of nanoparticles. By applying AFM technique, the true structure of nanoparticles can be revealed with very high resolution since the image is obtained by direct contact or tapping of the AFM tip on or over the particles surface.

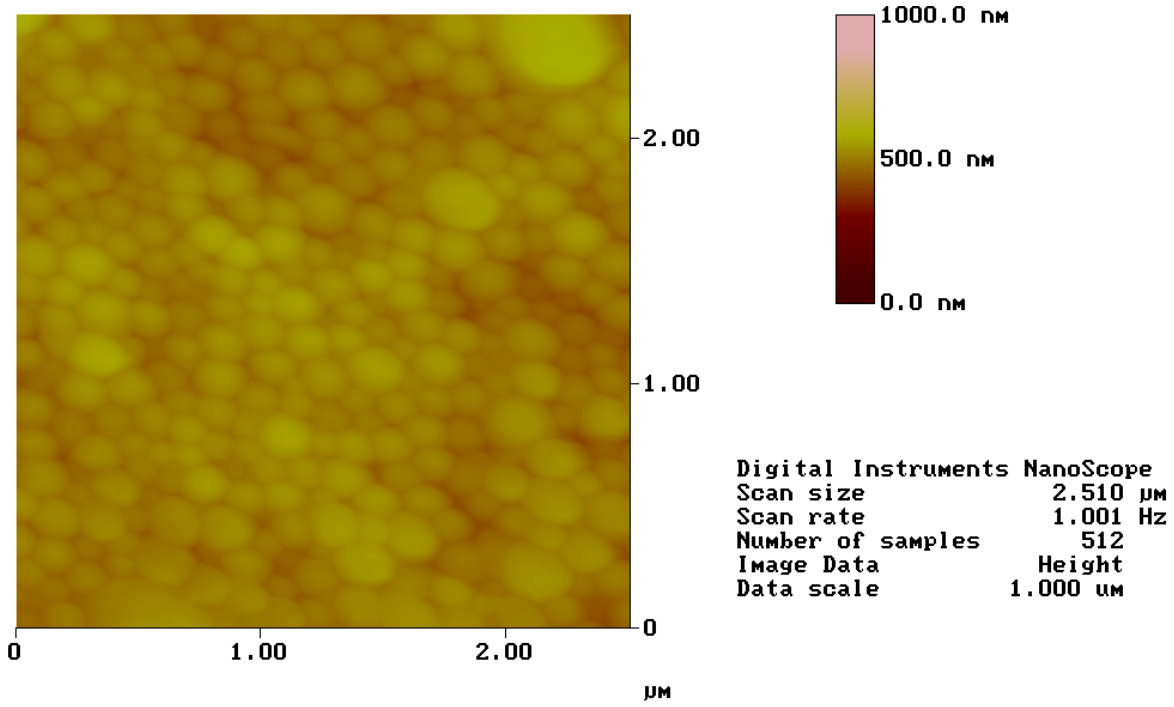


Figure 3. 8. AFM overview image of a layer of paclitaxel-loaded PLGA nanoparticles prepared with PVA as emulsifier.

There was no observed aggregation or adhesion of the nanoparticles (Fig. 3.8). AFM gives direct 3-D measurements of the surface structure of the samples. Further, using materials sensing modes such as lateral force and phase contrast, it is possible to differentiate the types of materials at a sample surface. Figure 3.9(a) shows the 3D image of a single paclitaxel loaded, PVA emulsified PLGA nanoparticle. Figure 3.9(b) shows closely observed surface morphology in a zoom-in image while Figure 3.9(c) shows the cross-section analysis of a single nanoparticle.

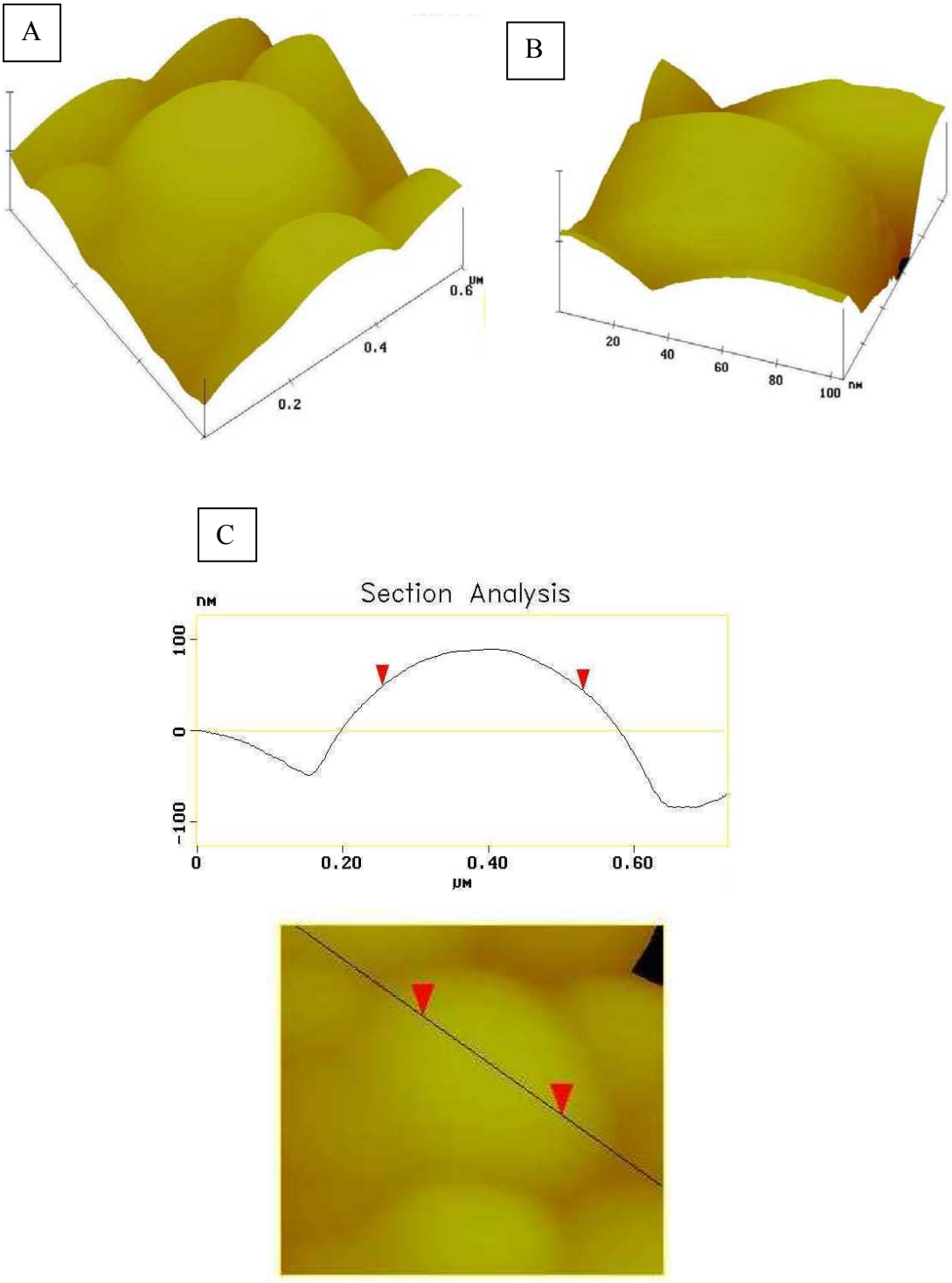


Figure 3. 9. AFM images: (A) 3D image; (B) close-up image; (C) cross-section and topography images of PLGA particles prepared with PVA as emulsifier.

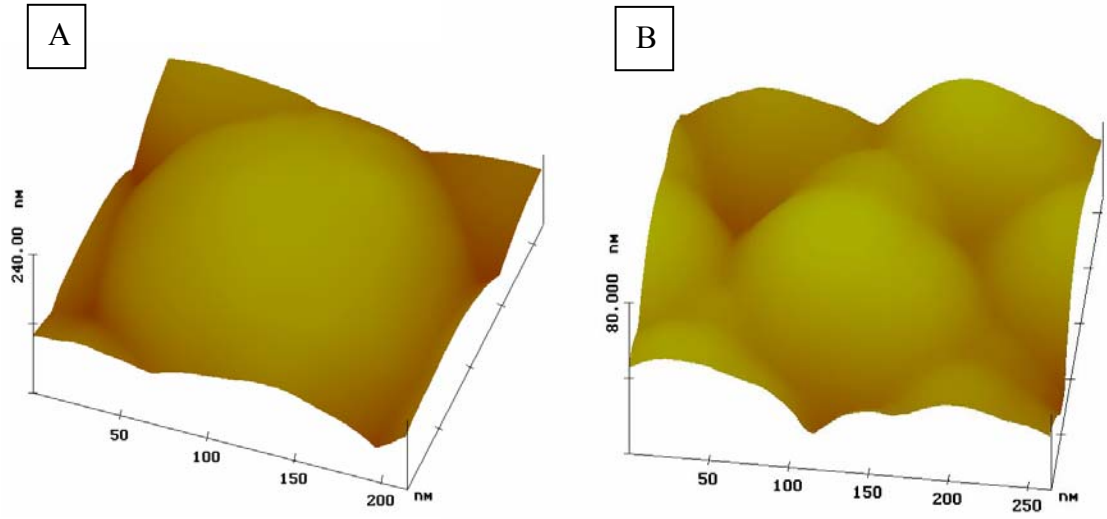


Figure 3. 10. AFM 3D images of paclitaxel loaded PLGA nanoparticles incorporating (a) TPGS; (b) DPPC.

AFM images of the nanoparticles fabricated with TPGS or DPPC were seemed to have a similar feature (Fig. 3.10) and smooth surface. AFM offers the capability of 3D visualization and both qualitative and quantitative information on many physical properties including size, morphology, surface texture and roughness. AFM investigation clearly substantiates the existence of complex topography of the nanoparticle surface, which contains micro-caves and pores (Fig. 3.11) rather than simply being smooth as shown in the conventional SEM images. Such a porous structure of the nanoparticle surface could explain a diffusion mechanism of the drug from the nanoparticles. This can be explained by the denser packing of TPGS or DPPC on the surface.

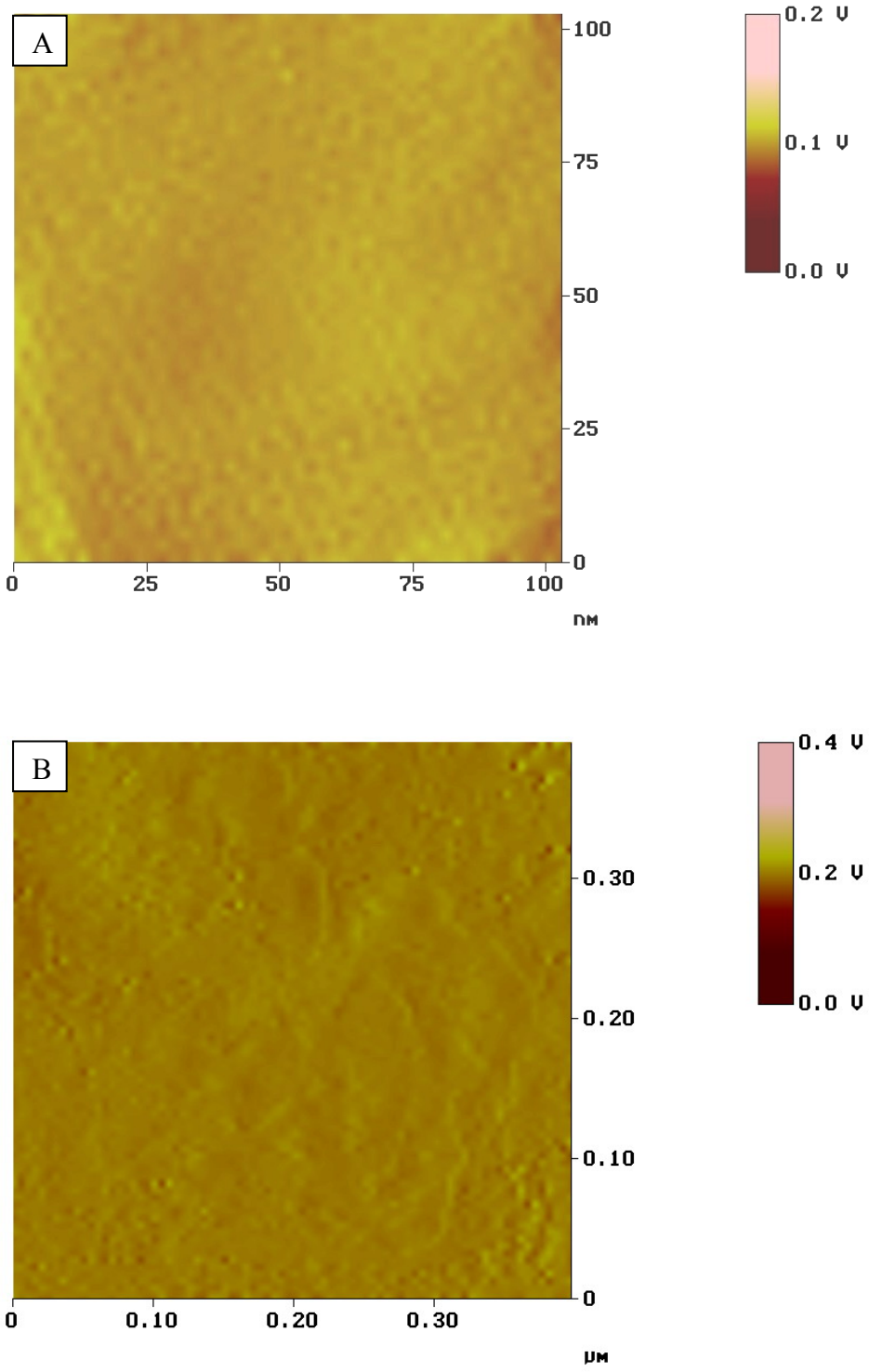


Figure 3. 11. AFM image clearly visualizing the complex topography of paclitaxel-loaded (A) TPGS- and (B) DPPC-incorporated PLGA nanoparticle surface.

3.3.4. Zeta potential analysis

Zeta potential can be quantified by tracking the charged particles when they migrate in a voltage field, as is done in a zeta potential analyzer. Since the particles in contact with a liquid acquire an electronic charge on their surface, zeta potential is a useful indicator of this surface charge property and can be employed as an index to the stability of the nanoparticles.

In most circumstances, the higher the absolute value of the zeta potential of the particles, the larger the amount of charge on their surface resulting in the stronger repellent interaction among the particles to overcome the natural tendency to aggregate and hence higher stability of the particles is achieved. Therefore, higher value of zeta potential implies more stable suspension and lower value points out the colloid instability. In the area of drug delivery studies, zeta potential analysis is often employed to investigate various systems such as nano- and microparticles, liposomes, micelles, etc.

The zeta potential values of the drug-loaded nanoparticles prepared with various emulsifiers/surface modifiers are shown in Figure 3.12. The negative zeta potential value implies negative charge on the surface and may be attributed to the presence of ionized carboxyl groups on the particles surface (Stolnik et al., 1995). The particle suspension is said to be stable if the zeta potential value is higher than 18. As the concentration of PVA changed, the zeta potential also changed and it was found to have the highest value at high PVA concentration of 8% (data not shown). However, the size of the particles and the effectiveness of the removal of excessive PVA from the suspensions also need to be taken into considerations. The stability and the size have to be compromised to achieve a better formulation for enhanced uptake and

bioavailability. The zeta potential was found to be strongly influenced by the emulsifier applied in the fabrication of particles.

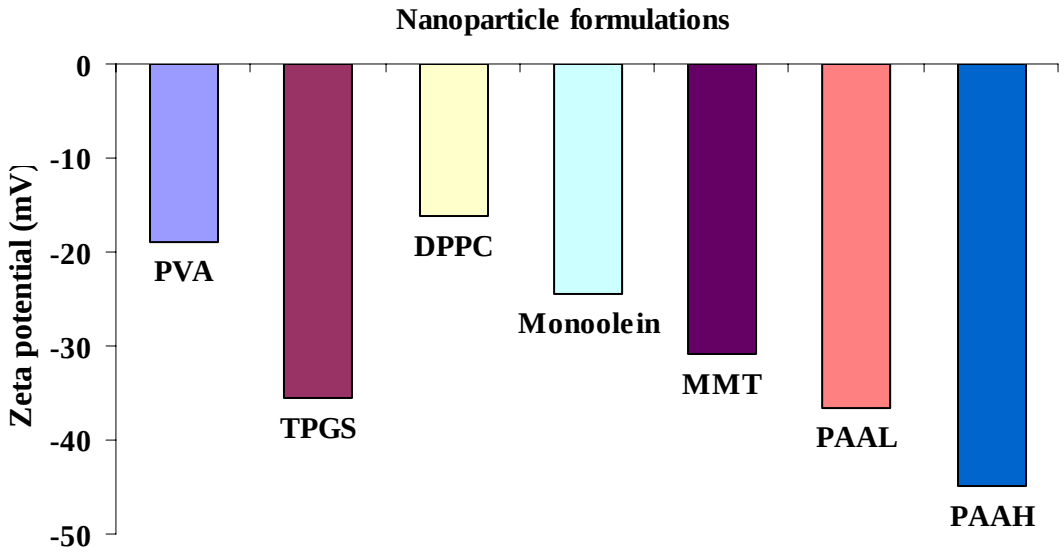


Figure 3. 12. Zeta potential analysis of various formulations of paclitaxel-loaded PLGA nanoparticles.

3.3.5. X-ray diffraction study

To determine whether paclitaxel was incorporated in the nanoparticles in its crystalline or amorphous form, an XRD study was conducted. Figure 3.13 shows the XRD patterns of free paclitaxel, pure TPGS and TPGS-incorporated PLGA nanoparticles with and without paclitaxel encapsulated. It appeared that paclitaxel was semi-amorphous and that PLGA (data not shown) and TPGS were amorphous which explained why the blank PLGA nanoparticles expressed the amorphous state. The XRD pattern of paclitaxel-loaded TPGS-incorporated PLGA nanoparticles clearly demonstrates that they consisted of a mixture of PLGA, TPGS and paclitaxel since the graphs of the blank nanoparticles and paclitaxel can be superimposed to form the

graph of the drug-loaded nanoparticles. In addition, these XRD patterns lead to the conclusion that the drug is present in the nanoparticles in an amorphous state since no crystalline diffraction pattern is distinguishable.

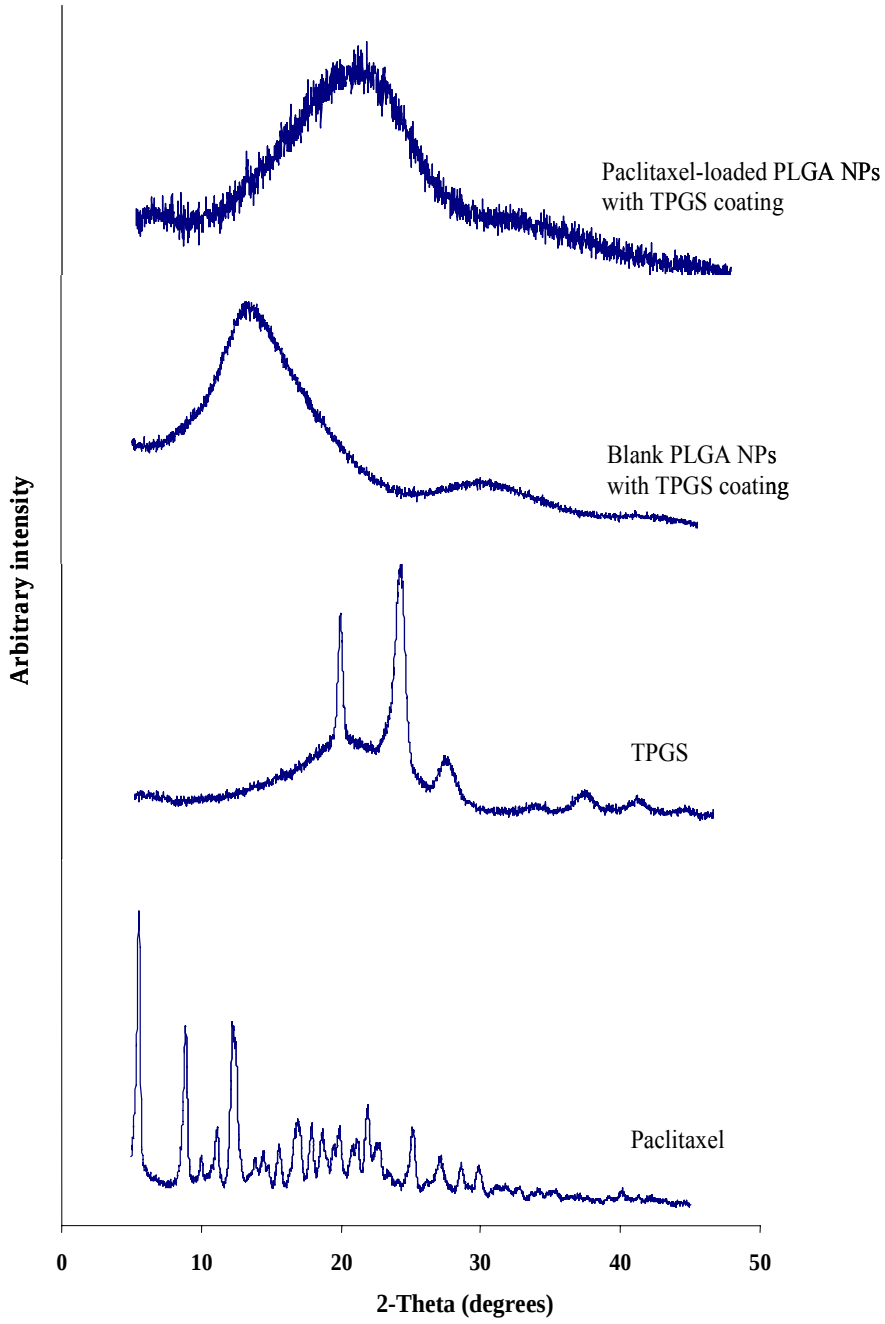


Figure 3. 13. XRD analyses of paclitaxel, TPGS, blank PLGA nanoparticles and paclitaxel-loaded PLGA nanoparticles with TPGS coating.

If the drug dissolves completely in the organic solvent phase, it may or may not form crystalline domain in the polymer matrix that makes up the nanoparticles. Microspheres with low active agent payloads (e.g., <20 wt %) have no crystalline domain as determined by differential thermal analysis. At higher payloads, crystallinity may appear. The drug loading in this work was 5% and the XRD patterns of drug-loaded PLGA nanoparticles, regardless of the incorporating material (PVA, TPGS or DPPC), expressed that the drug was present in an amorphous state in all those potential formulations (Fig. 3.14).

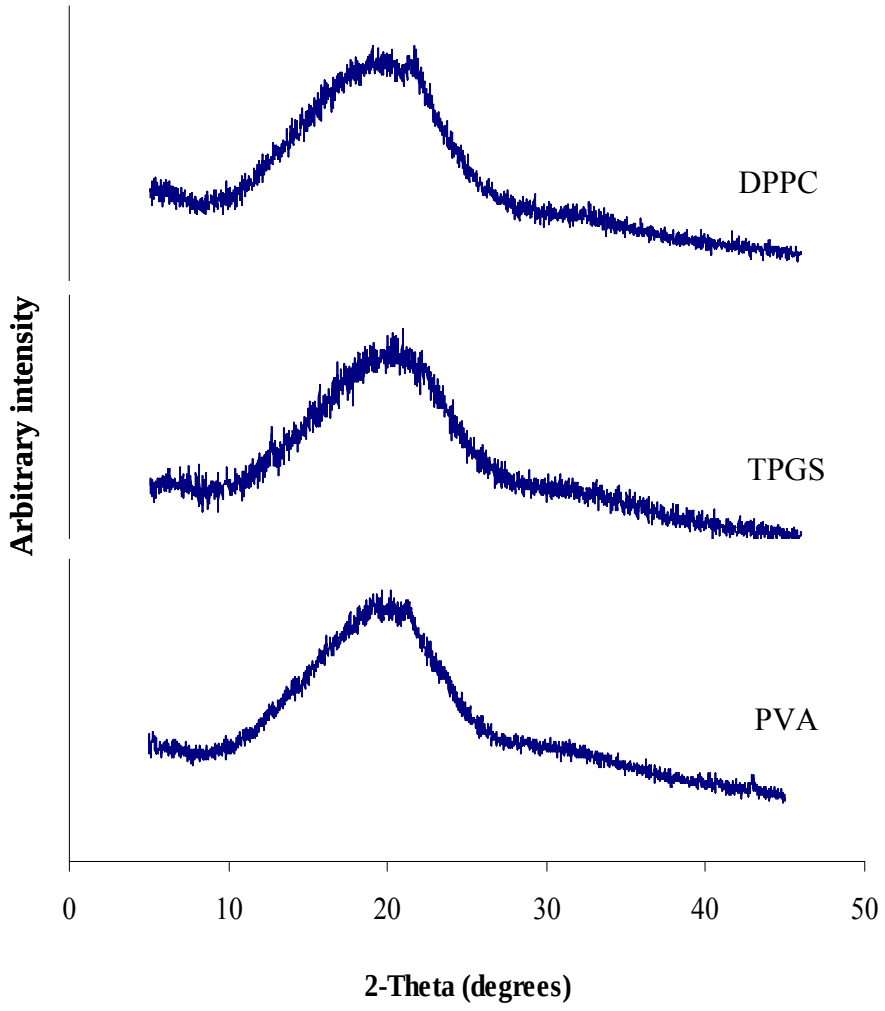


Figure 3. 14. XRD pattern of paclitaxel-loaded PLGA nanoparticles incorporating PVA, TPGS and DPPC.

3.3.6. *In vitro* paclitaxel release studies

The effect of emulsifiers on the *in vitro* release profiles of paclitaxel was presented in Fig. 3.15. There are three primary mechanisms of the drug release from PLGA microparticles: swelling, diffusion and degradation (Schwendeman et al., 1996). The mechanism of paclitaxel release from the formulated nanoparticles was found to be dominated by diffusion through tortuous paths in the polymer matrix. Since the degradation time of polymer is about 1-2 months, the release by polymer matrix erosion was not significant in this release period.

The nanoparticles formulated with TPGS, DPPC and MMT exhibited almost the same release pattern with higher initial burst and the accumulated amount of paclitaxel released in 31 days was about 70-75 %. High initial burst was attributed to the immediate dissolution and release of paclitaxel adhered on the surface and located near the surface of the nanoparticles. The nanoparticles incorporating PAA (both low and high Mw) and PVA possessed almost identical release pattern, especially for the first 15days, and their cumulative release of paclitaxel was 29-34% after 31 days. The faster drug release of the former group maybe due to the facilitation of the drug solubility by the additives (i.e. TPGS, DPPC and MMT) and hence more drug diffusion leading to the higher and faster drug release from the nanoparticles. Monoolein emulsified nanoparticles released about 46% of paclitaxel in 31 days.

The release profile can be optimized since the amount of emulsifier used in the preparation process plays a significant role in controlling the drug release profile (Feng and Huang, 2001; Mu and Feng, 2001). In general, similar release patterns, though the released amount varied according to the emulsifier used, were observed for

the nanoparticles prepared with different emulsifiers, providing slower and steady release rate after the initial burst.

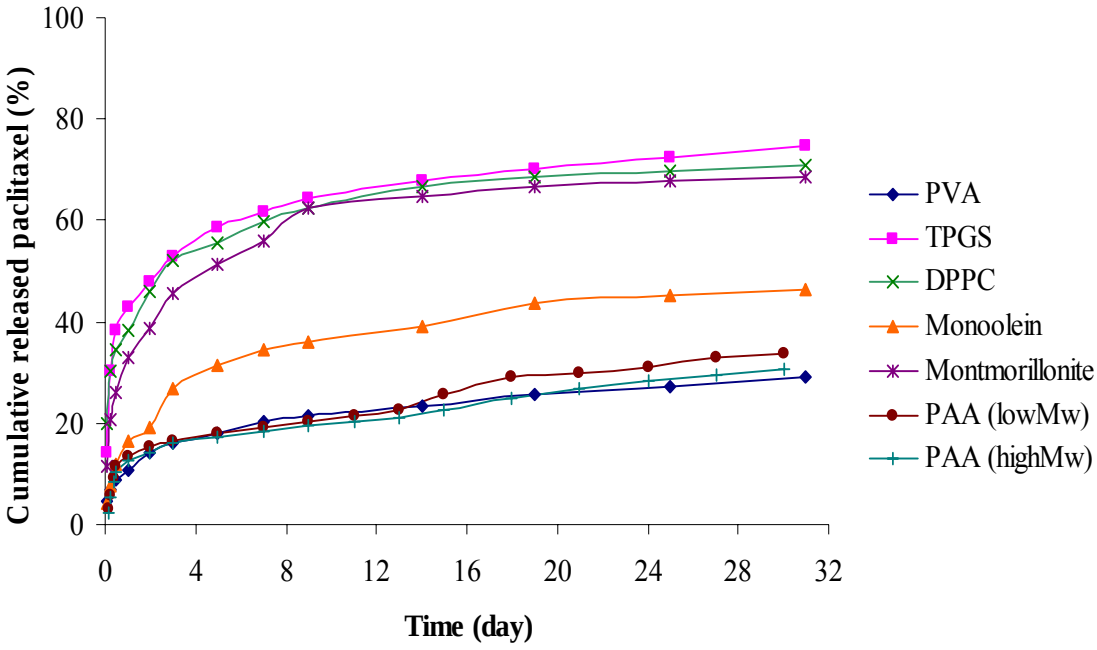


Figure 3. 15. Effect of emulsifier/additive on in vitro release of paclitaxel from nanoparticles.

3.3.7. *In vitro* degradation studies

It is important to understand the degradation profiles of the particles in order to develop successful drug delivery system providing suitable release profile. As can be seen from Fig. 3.16, erosion of the PLGA particles occurred only after the second week. This confirmed the above postulation of drug release by diffusion through the polymer matrix.

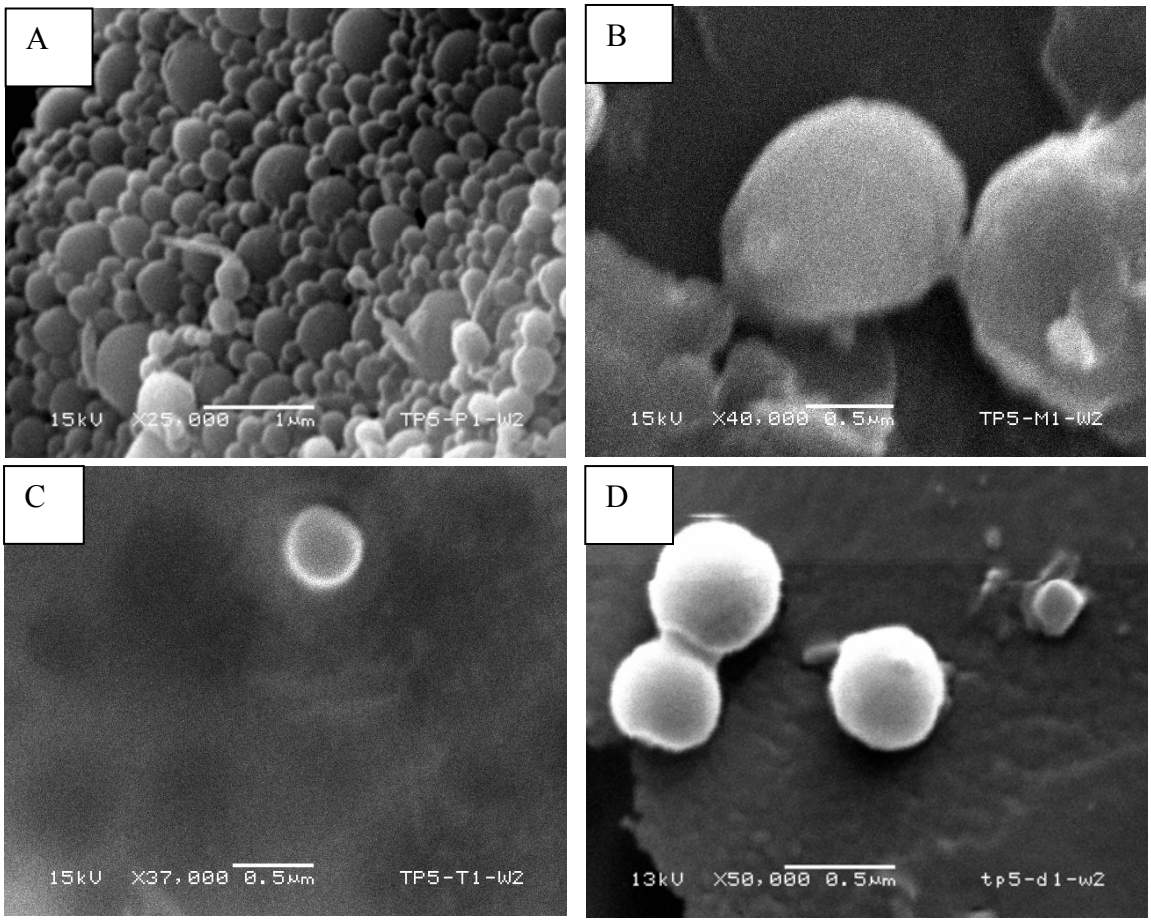


Figure 3. 16. Degradation profile of paclitaxel-loaded PLGA particles with: A) PVA; B) montmorillonite; C) vitamin E TPGS; D) DPPC after 2 weeks under the simulated physiological conditions.

For the amorphous PLGA particles, degradation in PBS solution was believed to occur in two stages. The first stage, water diffusion into the amorphous regions with random hydrolytic scission of ester bonds, occurred in the first two weeks of the study. The second stage started when most of the amorphous regions are degraded from the third week onwards, after which the hydrolytic attack progresses from the edge towards the centre of crystallites. Figure 3.17 shows the SEM images of degradation of nanoparticles over 4 and 8 weeks. At 4 weeks, the degradation has proceeded but the original morphology was still discernible. The continuing

degradation induced agglomeration of individual particles. At 8 weeks, the formulated nanoparticles became highly porous and have lost their original surface features and thus, they lost their mechanical integrity, as shown in Fig. 3.18. The nanoparticles now appeared to have started coalesce, appearing as an amorphous mass and their original morphology can no longer be seen.

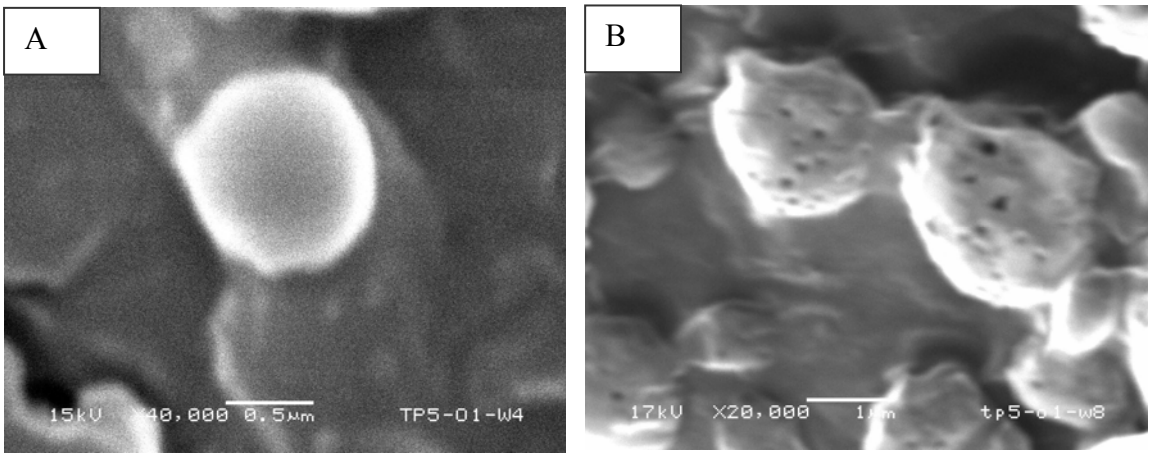


Figure 3. 17. Degradation profile of paclitaxel-loaded PLGA particles with monoolein as emulsifier: A) after 4 weeks; B) after 8 weeks.

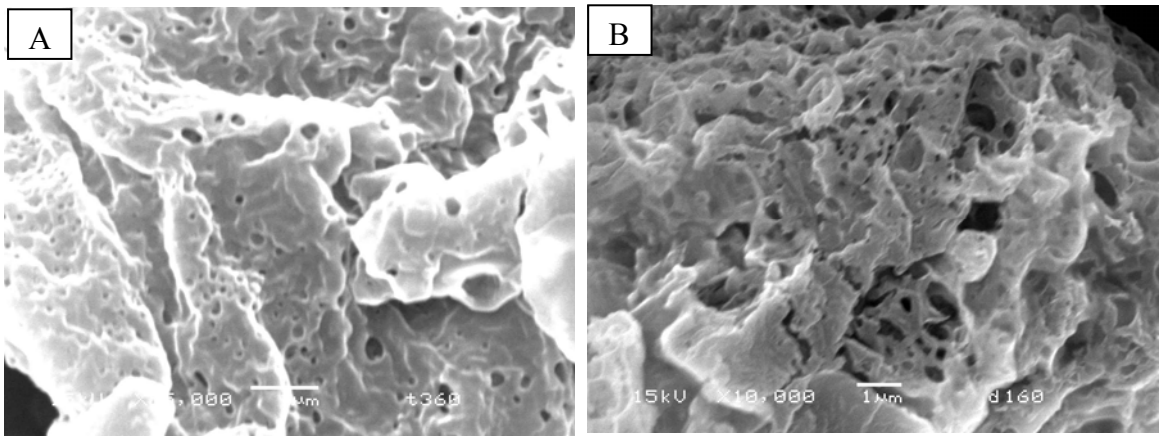


Figure 3.18. SEM images of paclitaxel-loaded PLGA particles incorporating A) TPGS and B) DPPC after 8 weeks in simulated physiological conditions at 37°C.

The effect of the degradation on the loss of molecular weight determined by GPC was shown in Fig. 3.19. These results indicated that the emulsifier has certain effect on degradation of nanoparticles and hence their molecular weight loss. Furthermore, it provided the quantitative evidence of the degradation behaviors observed in the qualitative SEM images.

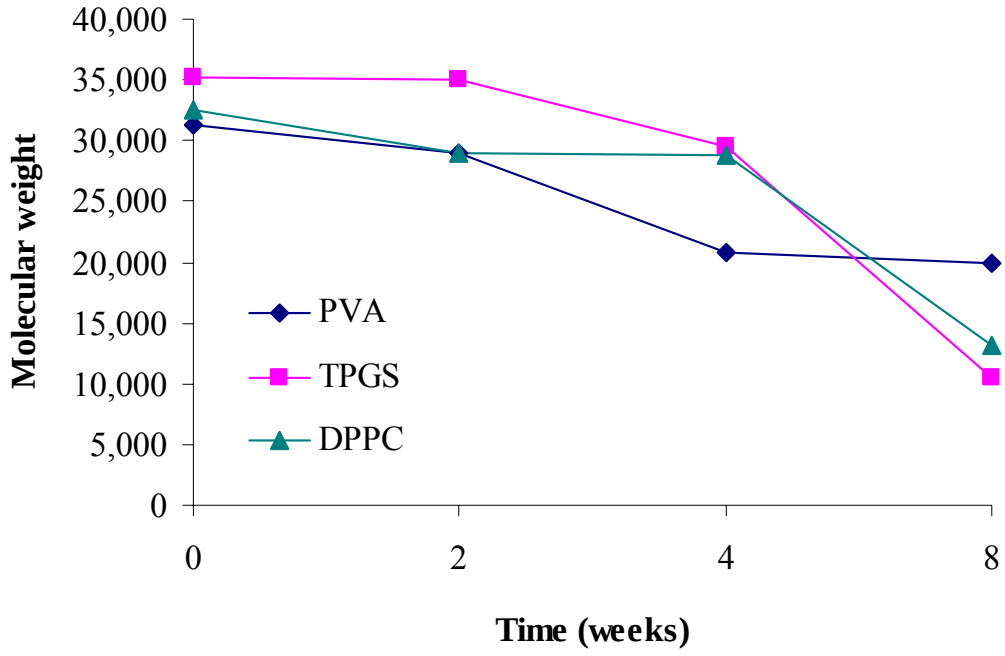


Figure 3. 19. Degradation profile of paclitaxel-loaded particles.

In the first stage of degradation of the semi-crystalline PLGA polymer, water uptake was basically governed empirically by the intrinsic hydrophilicity of the repeat units and by end-group effects. Since the end groups of PLGA are alkoxylic and carboxylic whose number is increased as molecular weight falls due to degradation, the essentially hydrophobic polymer becomes more hydrophilic. Water uptake was determined by two events. The first was simple diffusional ingress into the dry interior of the particles and in the absence of degradation this would occur to a level

that would be characteristic of the equilibrium swelling of this kind of material. However, these polymers are hydrolytically unstable and following, or even during this initial diffusional phase, the polymer can degrade and take up more water. In the second stage of degradation, water uptake was then through the highly porous surface of the particles as the amorphous regions were degraded. By then, a loss of nanoparticles integrity was inevitable due to the breakdown of the polymer matrix, which was reflected by the increased porosity on the surface. From the results presented here, it can be predicted that the morphology of the polymeric matrix should greatly influence the drug release profiles, because for both diffusion and degradation controlled mechanisms, the matrix morphology is a key factor.

3.4. Conclusion

Biodegradable polymeric nanoparticles were successfully prepared by oil-in-water emulsification/solvent evaporation process using both chemical and natural emulsifiers. A combination of the optimized parameters gives rise to a small, reproducible particle size with narrow size distribution and high encapsulation efficiency.

Comparing to chemical emulsifiers, natural surfactants such as polysaccharides, phospholipids, cholesterol and vitamin are preferred since they may have better performance in preparation and application of polymeric nanoparticles for clinical administration. Moreover, the natural emulsifiers DPPC and TPGS have much higher emulsifying efficiency comparing to the chemical emulsifier PVA. This implies that much lower amount of DPPC (about 1/33 of PVA) or TPGS (about 1/67 of PVA) is needed for the same formulation procedures. This is quite possible since TPGS and

DPPC have greater tendency to migrate to the surface of nanoparticles which has been supported by the XPS analysis of the surface chemistry (Feng et al., 2004). Furthermore, XRD analyses revealed that the drug was present in these nanoparticles in an amorphous state. The drug release profile and the degradation pattern are comparable for these TPGS and DPPC-incorporated nanoparticle formulations. Therefore, nanoparticle formulations incorporating TPGS and DPPC were selected as the most suitable formulations for further evaluations.

CHAPTER 4

EFFECTS OF PARTICLE SIZE AND SURFACE COATING ON CELLULAR UPTAKE OF POLYMERIC NANOPARTICLES FOR ORAL DELIVERY OF ANTICANCER DRUGS

4.1. Introduction

Oral delivery of anticancer drugs is a challenge, which has advantages over the current regime of chemotherapy by injection or infusion. Oral chemotherapy can provide a long-time, continuous exposure of the cancer cells to the anticancer drugs of a relatively lower thus safer concentration and thus give little chance for the tumor blood vassals to grow, resulting in much better efficacy and fewer side effects than the current intermittent chemotherapy could do. Oral chemotherapy is convenient and thus preferred by the patients, which can greatly improve the quality of life of the patients. This is especially important for the patients with advanced or metastatic cancer. Oral chemotherapy can eventually promote a new concept of chemotherapy: "chemotherapy at home" (Feng and Chien, 2003; Feng et al., 2004; Bottomley, 2002; Ajani and Takiuchi, 1999; Demario and Ratain, 1999). Unfortunately, most anticancer drugs are not bioavailable due to their pure solubility, stability and permeability, i.e. orally administrated anticancer drugs have little chance to get into the blood system and thus reach the tumor site. The reason has been under intensive investigation and it has been found that the orally administrated anticancer drugs would be eliminated by the first metabolic process with cytochrome P450 and by the efflux pump of P-glycoproteins (P-gp) (Malingré et al., 2001; Sparreboom et al., 1997). Medical

solutions, which are currently being developed in pharmaceutical companies, usually propose to apply P450/P-gp suppressors such as cyclosporin A to make oral chemotherapy feasible. However, the P450/P-gp suppressors would fail the immune system of the patients and thus may cause complex medication to the patients. Also, most P450/P-gp suppressors may have side effects and/or difficulties in formulation of their own (Malingré et al., 2001; Van Zuylen et al., 2000; Terwogt et al., 1998; Bonduelle et al., 1996). Nanoparticles of biodegradable polymers may provide an alternative solution for oral delivery of anticancer drugs across the gastrointestinal barrier due to their extremely small size and their appropriate surface coating to escape from the recognition by P450/P-gp (Maincent et al., 1986; Florence et al., 1995; Wang et al., 1996; McClean et al., 1998; Suh et al., 1998; Delie, 1998; Matsumoto et al., 1999; Jung et al., 2000; Feng and Huang, 2001; Mu and Feng, 2002; Mu and Feng, 2003).

It has been found that the size of the nanoparticles plays a key role in their adhesion to and interaction with the biological cells. The possible mechanisms for the particles to pass through the gastrointestinal (and other physiological) barriers could be (1) paracellular passage – particles "kneading" between intestinal epithelial cells due to their extremely small size (<50 nm); (2) endocytotic uptake – particles adsorbed by intestinal enterocytes through endocytosis (particles size < 500 nm); and (3) lymphatic uptake – particles adsorbed by M cells of the Peyer's patches (particle size < 5 microns) (Sanders and Ashworth, 1961; Lefevre et al., 1978; Florence et al., 1995). Also, coating the particles by appropriate bioadhesive materials such as polyvinyl alcohol (PVA), poly(ethylene glycol) (PEG), vitamin E TPGS, etc can greatly improve their adhesion to and adsorption into the intestinal cells as well as the

ability to escape from the multi-drug resistance pump proteins (Feng and Huang, 2001; Mu and Feng, 2002; Mu and Feng, 2003).

Vitamin E succinated polyethylene glycol 1000 (Vitamin E TPGS or simply TPGS) is a water soluble derivative of vitamin E, which has been found to be an excellent emulsifier/solubilizer/absorption enhancer of high emulsification efficiency and cellular adhesion (Mu and Feng, 2002; Mu and Feng, 2003). Fischer (2002) reported that co-administration of vitamin E TPGS increased the oral bioavailability of cyclosporine A in healthy dogs by non-compartmental pharmacokinetic analysis. Vitamin E TPGS acts as a reversal agent for P-glycoprotein mediated multidrug resistance and inhibits P-gp mediated drug transport (Dintaman and Silverman, 1999; Rege et al., 2002). Vitamin E TPGS can also enhance the absorption flux of amprenavir, a HIV protease inhibitor, by increasing its solubility and permeability (Yu et al., 1999).

Phospholipid, 1,2-dipalmitoylphosphatidylcholine (DPPC) is used as as an additive in our drug delivery formulation as it can improve the performance of the PLGA microspheres in blood flow (Garti, 1999), enhance the pulmonary absorption of peptides and proteins (Zhen et al., 1995), reduce phagocytic uptake of the microparticles (Evora et al., 1998), and impart better emulsifying effects and higher encapsulation of drug in the nanoparticles (Feng and Huang, 2001). Phospholipids exhibit high potential of application in drug delivery formulations.

The present study evaluated the cellular uptake of polymeric nanoparticles by using Caco-2 cells as an *in vitro* model with the aim to apply nanoparticles of biodegradable polymers for oral chemotherapy with emphasis on possible effects of particles size and particle surface coating on the cellular uptake of the drug loaded nanoparticles.

Caco-2 cells are an established epithelial cell line derived from a human colon adenocarcinoma that undergoes enterocyte differentiation in culture (Pinto et al., 1983). Confluent Caco-2 cell monolayers form tight junctional complexes, exhibit dome formation and electrical properties similar to those of the intestinal epithelium. This cell line has been suggested to possess attributes that make it a suitable *in vitro* model system for the investigation of transport across the small intestinal epithelium (Hidalgo et al., 1989) and particle uptake into human intestine (Artursson et al., 1990; Desai et al., 1997; McClean et al., 1998; Delie, 1998; Boudad et al., 2001). In the present study, fluorescent marker coumarin-6 was encapsulated in the poly (lactic-co-glycolic acid) (PLGA) nanoparticles to visualize the cellular uptake of the nanoparticles, which were manufactured by a modified solvent extraction/evaporation technique with PVA or vitamin E TPGS or phospholipids DPPC as emulsifier. To investigate the quantitative effects of the particle size on the cellular uptake of nanoparticles, commercially available fluorescent polystyrene nanoparticles were also used. The nanoparticles were characterized by laser light scattering (LLS) for their size and size distribution, scanning electron microscopy (SEM) for their surface morphology and zeta-potential measurement for their surface charge. The in vitro fluorescence release from the nanoparticles was measured by high performance liquid chromatography (HPLC). Cell uptake of the coumarin-6 loaded nanoparticles and intracellular location of the nanoparticles of various size and surface coating were investigated by confocal laser scanning microscopy (CLMS), cryo-SEM and transmission electron microscopy (TEM). It was found that vitamin E TPGS coated PLGA nanoparticles have advantages in favor of cellular uptake over those of other formulations and are thus of great potential for oral chemotherapy.

4.2. Experimental methods

4.2.1. Materials

Fluorescent polystyrene nanoparticles were purchased from Duke Scientific (CA, USA). Fetal bovine serum (FBS) was received from Gibco (Life Technologies, AG, Switzerland). All the remaining materials are the same as in section 3.2.1. Ultrapure water (Millipore, Bedford, MA, USA) was used throughout the experiment.

4.2.2. Preparation of nanoparticles

For the in vitro particle uptake studies, fluorescence labeled nanoparticles were also successfully prepared by a modified solvent extraction/evaporation method (single emulsion), as mentioned in the section 3.2.2, using PVA, Vitamin E TPGS and phospholipids DPPC as emulsifier. Coumarin-6 was encapsulated in the nanoparticles as the same manner of drug loading. The polymer solution contained 0.05% (w/v) coumarin-6 as fluorescent marker in preparation of fluorescent nanoparticles. Formulation optimization was pursued to obtain nanoparticles of desired physicochemical properties.

4.2.3. Characterization of nanoparticles

4.2.3.1. Size and size distribution

Nanoparticle size and size distribution were determined by laser light scattering with particle size analyzer (90 Plus, Brookhaven Inst, Huntsville, NY, USA) at a fixed

angle of 90° at 25°C. The data reported in Table 4.1 represent the average of 5 measurements.

4.2.3.2. Surface morphology

Morphology of the formulated nanoparticles was observed by scanning electron microscopy (SEM, Jeol JSM 5600LV) after preparing the sample with a platinum coating by a sputter coater (JFC-1300, Jeol, Tokyo) for 40 seconds in a vacuum at a current intensity of 40 mA.

4.2.3.3. Surface charge

Zeta potential of nanoparticles, an indicator of surface charge and stability of dispersion, was determined by a zeta potential analyzer (Zeta Plus, Brookhaven Instruments, Huntsville, NY) and the mean value of 10 readings was reported.

4.2.4. In vitro release of fluorescent markers from nanoparticles

Coumarin-6 loaded nanoparticles were dispersed in transport buffer at pH 7.4, which was used to simulate physiological fluid. The buffer solution was kept in an orbital shaker at constant gentle shaking of 110 rpm at 37°C. At pre-determined time intervals, the suspensions were centrifuged at 11,000 rpm for 8 min. The precipitated particles were re-suspended in fresh buffer and placed back into the shaker. The supernatant containing released fluorescent marker was then analyzed to determine the percentage release of the fluorescent markers from the nanoparticles.

4.2.5. Cell culture

In the present study, Caco-2 cells of passages between 24 and 30 were used. Caco-2 cells were cultured in MEM medium with 1.5 mM L-glutamine, supplemented with 20% fetal bovine serum (FBS), 1mM sodium pyruvate, 1.5 g/L of sodium bicarbonate and 1% penicillin-streptomycin solution. Cells were seeded at 6.4 x 10^4 cells/cm^2 on the 96-well black plates with transparent bottom (Costar, IL, USA) for quantitative uptake experiments or on the Lab-Tek® chambered cover glasses (Nalge Nunc, IL, USA) for confocal microscopy. Cells were cultured as a monolayer at 37°C in a humidified atmosphere containing 5% CO_2 and medium was replenished every other day.

4.2.6. Nanoparticle uptake by Caco-2 cells

4.2.6.1. Quantitative studies

Caco-2 cells were seeded in 96-well black plates (Costar, IL, USA) and incubated until they formed a confluent monolayer. Upon reaching confluence, the culture medium was replaced by transport buffer (Hank's balanced salt solution, HBSS, pH 7.4) and pre-incubated at 37ºC for 30 min. After equilibration, cell uptake of nanoparticles was initiated by exchanging the transport medium with 100 µL of specified nanoparticle suspension (100 µg/ml to 500 µg/ml in HBSS) and incubating the cells at 37°C for 0.5 to 4 h. The experiment was terminated by washing the cell monolayer three times with phosphate-buffered saline (PBS, pH 7.4) to eliminate excess particles which were not entrapped by the cells. Cell membrane was permeabilized with 0.5% Triton X-100 in 0.2 N NaOH solution to expose the internalized nanoparticles for the quantitative measurement. Cell-associated

nanoparticles were quantified by analyzing the cell lysate in a Genios microplate reader (Tecan, Männedorf, Switzerland, λ_{ex} 430 nm, λ_{em} 485 nm). Uptake was expressed as the percentage of fluorescence associated with cells versus the amount of fluorescence present in the feed solution.

4.2.6.2. Qualitative studies

4.2.6.2.1. Confocal laser scanning microscopy

Caco-2 cells were seeded on Lab-Tek® chambered cover glasses (Nalge Nunc International, Naperville, IL, USA) and incubated at 37°C in 95 % air and 5 % CO_2 environment until cells were about 70 % confluent. On the day of experiment, the growth medium was replaced by HBSS (pH 7.4). After equilibration with HBSS at 37ºC for 30 min, the buffer was replaced with nanoparticle suspension (250 µg/ml in HBSS) and then the monolayers were further incubated for 1 hr or 2 hr. At the end of experiment, the monolayers were washed 3 times with fresh pre-warmed transport buffer to eliminate excess nanoparticles which were not attached to the cells. Cells were then fixed with 70% ethanol and the nuclei were stained by propidium iodide (PI). The samples were mounted in the fluorescent mounting medium (Dako, CA) until examination was performed by the confocal laser scanning microscope (Zeiss LSM 410, Germany) equipped with an imaging software, Fluoview FV300.

4.2.6.2.2. Cryo-scanning electron microscopy (Cryo-SEM)

The cellular internalization of nanoparticles was confirmed by cryo-SEM. Caco-2 cells of passage 30 were incubated with nanoparticle suspension (250 µg/ml in HBSS, pH 7.4) for 1 hour and then the excess nanoparticles were washed away with pre-

warmed PBS (pH 7.4) for 3 times. Cells were fixed by using 2.5% glutaraldehyde solution and were plunged frozen in nitrogen sludge (-194°C). The specimen was transferred to the cryo-preparation chamber of a cryo-system attached to a Philips XL30 scanning electron microscope. The temperature was raised to -95°C. The specimen was then fractured and etched for 15 min. The frozen specimen was sputter-coated with approximately 5 nm of platinum, introduced onto the specimen stage of the SEM at -130°C and examined at 5-10 kV accelerating voltage.

4.2.6.2.3. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) of Caco-2 cells treated with nanoparticles was performed by negative staining. Briefly, at the end of 1 hr incubation with nanoparticles (250μg/ml), Caco-2 cells were washed, pre-fixed with 2.5% glutaraldehyde and 2% paraformaldehyde solution, post-fixed with 1% osmium tetroxide, dehydrated with a series of alcohols and infiltrated with resin. The resin sample block was trimmed, thin-sectioned to thickness of 70nm, and collected on formvar-coated copper grids. Before examining under the TEM, these grids were stained by uranyl acetate and lead citrate, followed by blotting with a filter paper and air-drying. Samples were examined in Philips CM10 at 200 kV.

4.3. Results and discussion

4.3.1. Physicochemical properties of nanoparticles

4.3.1.1. Size and size distribution

The particle size, polydispersity index, and zeta potential of the fluorescent nanoparticles are presented in Table 4.1. The nanoparticles formulated in this study

were found to be in the size range of 200-500 nm. The light scattering measurement of particle size agreed well with the measurement given by the SmileView software from SEM micrographs.

Table 4.1. Characteristics of fluorescent PLGA nanoparticles coated with PVA or vitamin E TPGS and standard fluorescent polystyrene nanoparticles

Sample Group	Emulsifier (w/v)	Size ± SD (nm)	Polydispersity	Zeta Potential (mV)
PVA	2 %	262 ± 8.8	0.133	-18.4
TPGS	0.03 %	295 ± 15	0.201	-29.7
DPPC	0.06 %	263 ± 3.9	0.046	-16.1
200nm PS	-	201 ± 2.3	0.036	-36.8
500nm PS	-	498 ± 8.4	0.136	-29.2
1000nm PS	-	1007 ± 35	0.125	-27.5

* Polydispersity = the size distribution width

4.3.1.2.Morphology of nanoparticles

The scanning electron microscopic images of the drug-loaded nanoparticles revealed their regular spherical shape (Fig. 4.1). Generally, their surface morphology was smooth without any noticeable pinholes or cracks within the conventional SEM resolution. The size distribution of all nanoparticles was unimodal with diameters in the total range of 150–500 nm and a mean diameter of 200–300 nm as confirmed by the laser light scattering measurement.

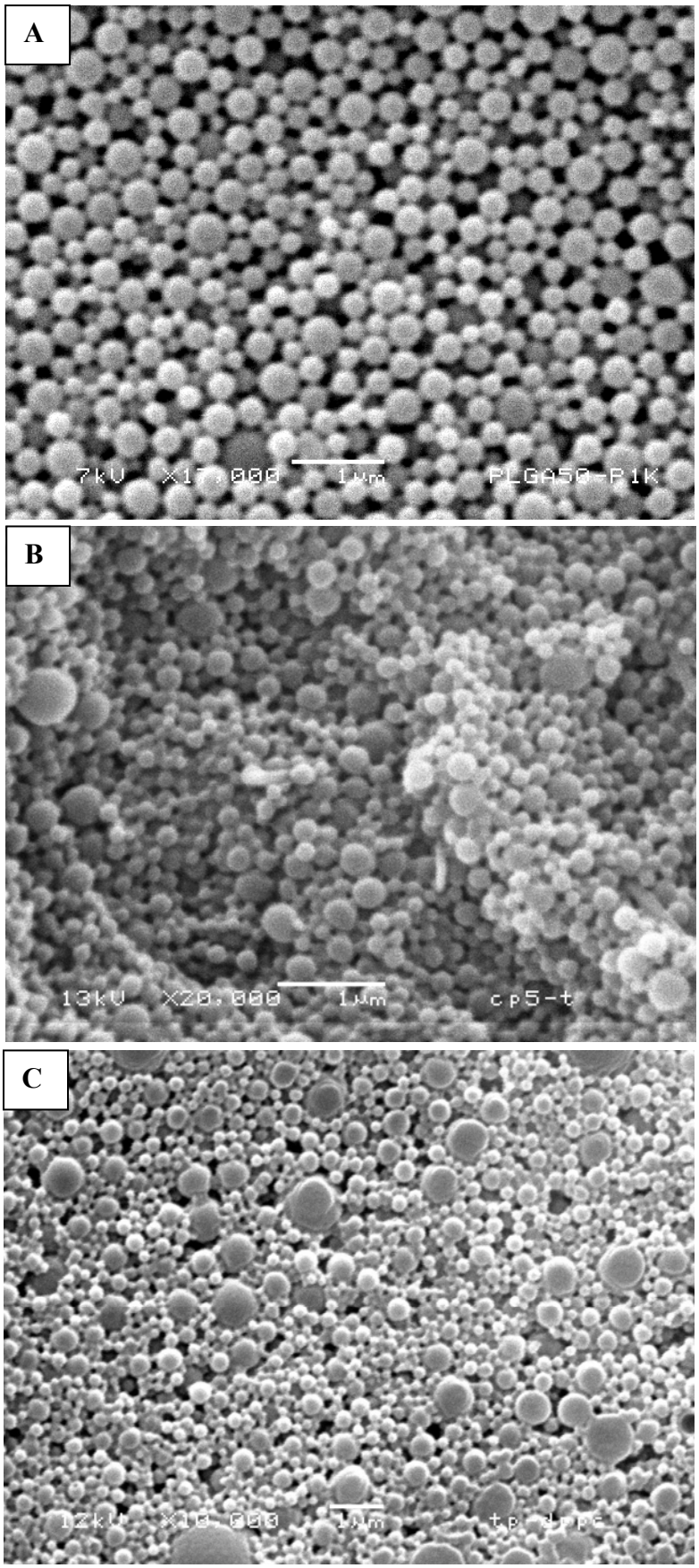


Figure 4. 1. SEM images of coumarin 6-loaded PLGA particles coated with PVA (A); vitamin E TPGS (B); and DPPC (C) (bar = 1 µm).

4.3.1.3.Surface charge of nanoparticles

The zeta potential was strongly influenced by the emulsifier used in the fabrication process of the nanoparticles. The nanoparticles in the present study were found stable in dispersion state, possessing high absolute values of zeta potential and having negative surface charges (Table 4.1).

4.3.2. In vitro fluorescent marker release

The use of fluorescent markers in nanoparticle visualization can lead to misinterpretation of nanoparticle uptake data due to the leaching or dissociation of fluorescent markers into the released medium (Suh et al., 1998) and hence subsequently into the cells. Neither could fluorometric analysis differentiate between intracellular and surface located particles, nor determine whether fluorescence detected was due to the cell-associated particles or the fluorescence released from the particles in the medium which was subsequently taken up by the cells. Thus, in vitro release study of fluorescence from the nanoparticle specimen was conducted to confirm the results obtained mainly due to the cell-associated nanoparticles but not from the released fluorescence in the medium. From Fig. 4.2, it can be found that the coumarin-6 was released only 3.75% and 2.55% from the TPGS and PVA nanoparticles, respectively, over 24 hr incubation time (Fig. 4.2), which was considered negligible in comparison with the nanoparticle uptake outcome of the Caco-2 cells. It is thus reasonable to assume that most of the coumarin-6 was associated in the nanoparticles and the fluorescence measured from the uptake samples mainly reflects the cellular associated fluorescent nanoparticles but not the released fluorescence in the medium.

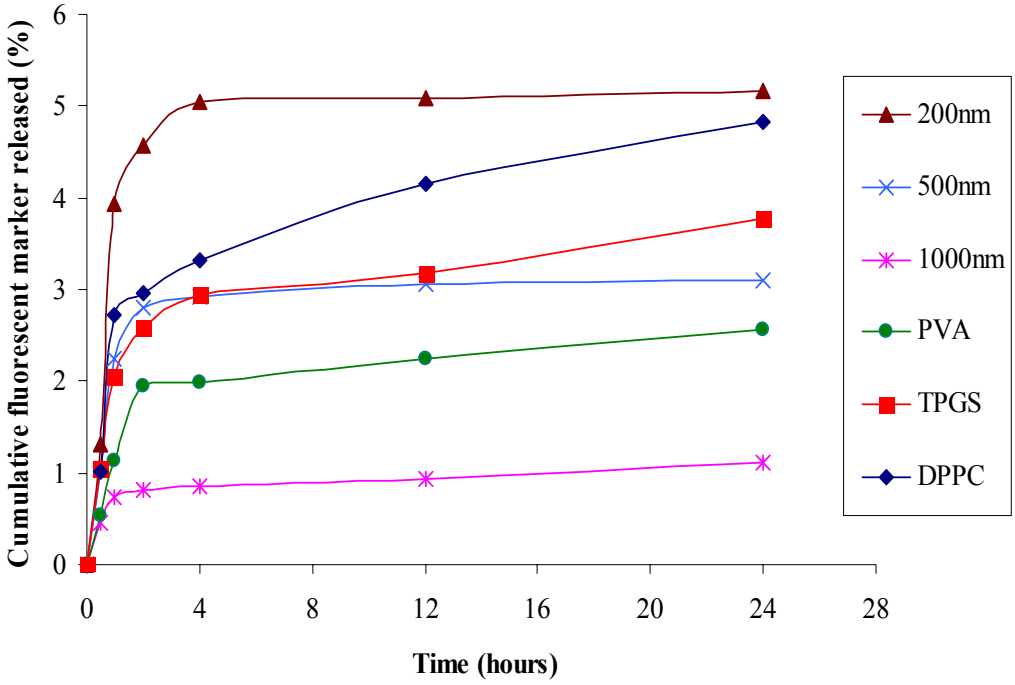


Figure 4. 2. In vitro release profiles of fluorescence from standard fluorescent polystyrene nanoparticles of 200nm, 500nm, 1,000nm diameter and PLGA nanoparticles coated with PVA, vitamin E TPGS, or phospholipids DPPC respectively. Data represents average value of triplicates.

4.3.3. Cell uptake of nanoparticles

In order to study cellular uptake of nanoparticles *in vitro* or *in vivo*, the use of fluorescently or radioactively labeled nanoparticles is the most common experimental approach found in the literature. Fluorescent labeling was chosen for the present study to avoid exposure of the samples to radioactive materials. Fluorescent labeling makes cellular uptake of nanoparticles readily detectable by fluorescence microscopy or CLSM. The extent of particle uptake can then be determined by flow cytometry, fluorometry, or quantitative extraction of the markers from the cells. Due to its similar structural and functional differentiation to mature enterocytes, the Caco-2 monolayer model is an established *in vitro* tool to evaluate the intestinal permeability and metabolism of drugs (Artursson, 1991; Yee, 1997). Reasonable correlations could be

established between *in vivo* data and data obtained in Caco-2 monolayers (Schakenraad, 1996). Thus, Caco-2 monolayers were used to study the various effects on cellular uptake of nanoparticles. The present study demonstrates that the Caco-2 cell uptake of nanoparticles is influenced by various parameters such as particle size, incubation time, particle concentration, surface properties of the particles, etc. TPGS coated nanoparticles are expected to increase their circulation time and internalization efficiency, and thus, to effectively improve the bioavailability of the drugs encapsulated in the nanoparticles.

4.3.3.1. Effect of particle surface coating, incubation time and temperature

Development of PLGA nanoparticles as an efficient delivery system would depend on their efficient internalization into, and sustained retention inside the cells. Here, surface properties of delivery system play an important role. Vitamin E TPGS coated PLGA nanoparticles were found to have improved the uptake of the nanoparticles by 3.9-folds, 4.4-folds and 6.1-folds over that of 200 nm, 500 nm and 1000 nm nude PS particles, respectively, and 1.4-folds over that of the PVA coated PLGA nanoparticles (Fig. 4.3). Moreover, the cell uptake of the DPPC coated PLGA nanoparticles was found to achieve the comparable uptake efficiency to that of TPGS nanoparticles. The PVA coated PLGA nanoparticles enhanced the uptake by 2.9-folds over that of the PS nanoparticles of about the same particle size, indicating that the polymer material of nanoparticles also contributes to the efficiency of cellular uptake. These results can thus support the assumption that the nanoparticle surface in contact with biological fluids, cells, or cellular components can be modified to provide favorite interactions with the cells (Zauner et al., 2001).

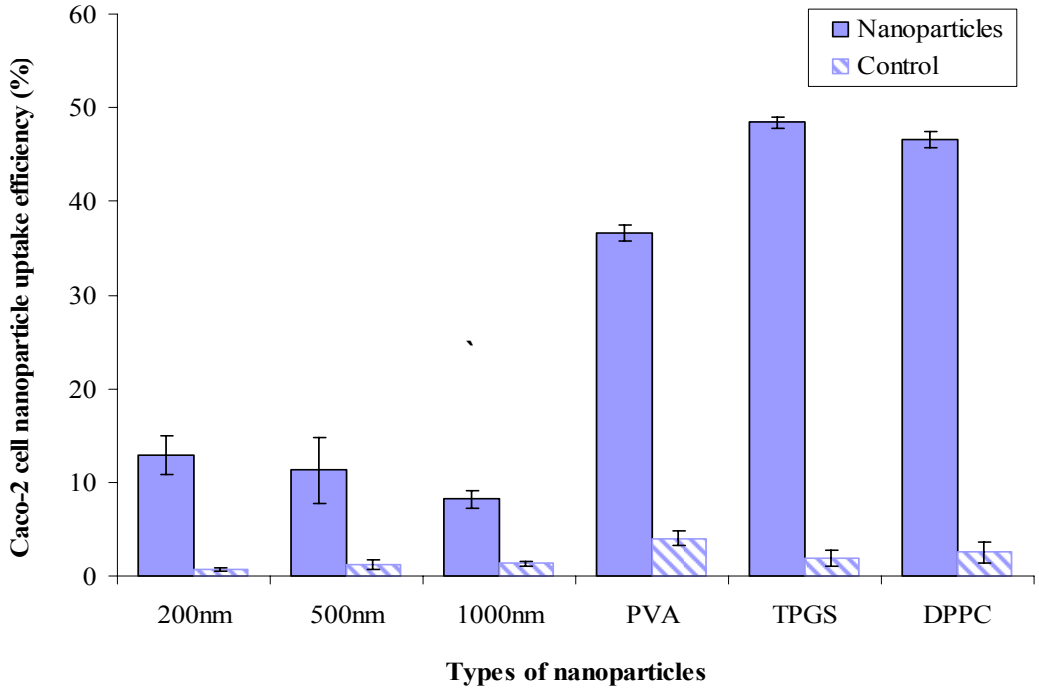


Figure 4. 3. Cellular uptake efficiency of standard fluorescent polystyrene nanoparticles of 200nm, 500nm, 1,000nm diameter and PLGA nanoparticles coated with PVA or vitamin E TPGS or DPPC, respectively, which is measured after 2 hours incubation with Caco-2 cells at 37°C. The control is the cellular uptake of coumarin-6 released from the nanoparticles under *in vitro* conditions and incubated with Caco-2 cells. Data represents mean ± SD, n=4.

Figure 4.4 shows that the uptake of nanoparticles by the Caco-2 cells at 37°C increased with the incubation time over 4 hr period. However, uptake of the nanoparticles by Caco-2 cells had no significant further increase beyond 2 hr incubation period since the curves showed a plateau effect. That could be due to the limited saturation level, which supports the claim by Desai (1997).

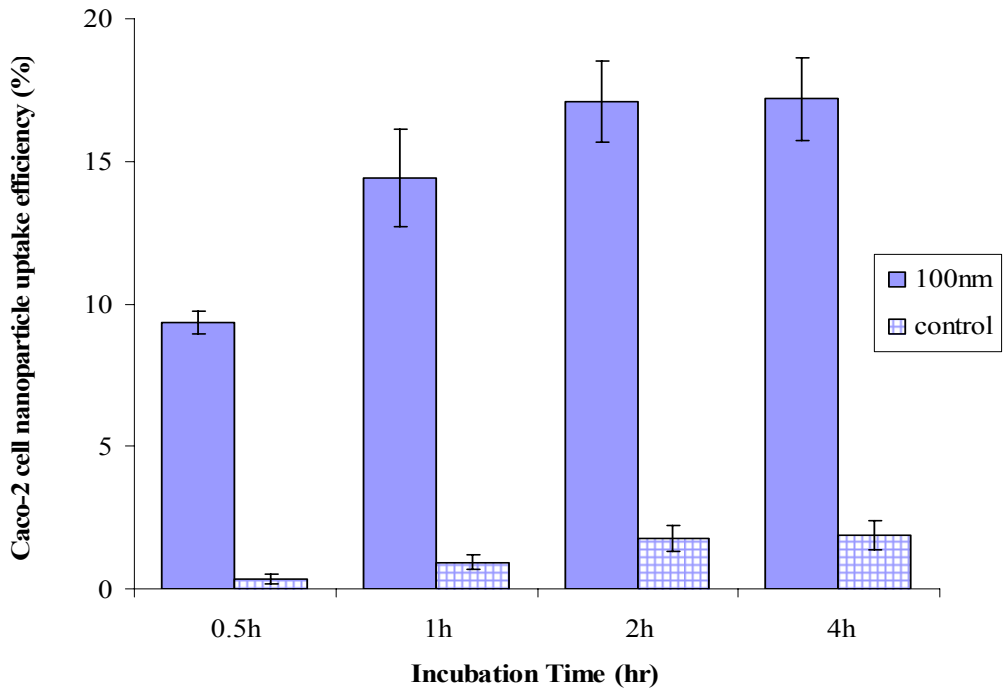


Figure 4. 4. Time courses for the Caco-2 cell uptake profile of fluorescent polystyrene nanoparticles of 100 nm cultured with nanoparticle concentration of 250 µg/mL at 37°C. The control is the cellular uptake of the coumarin-6 released from the nanoparticles under *in vitro* conditions and incubated with Caco-2 cells at the same conditions. Data represents mean ± SD, n = 4.

There was, in another series of experiment, a significant reduction in nanoparticle uptake by the Caco-2 cells at 4°C (data not shown), with the uptake being reduced to 25% to 46% of that at 37°C at equivalent particle concentration and incubation time. This suggests that the nanoparticle uptake by the Caco-2 cells could be due to the energy-dependent endocytic process.

4.3.3.2.Effect of particle size and concentration

Nanoparticles for chemotherapy need to be absorbable to cells with a sufficiently high rate and extent. It has been proposed that the size of the particles plays a key role in their adhesion to and interaction with the biological cells (Foster et al., 2001). It is, in

general, assumed that particles up to about 100–200 nm can be internalized by receptor-mediate endocytosis, while larger particles have to be taken up by phagocytosis (Couvreur and Puisicux, 2003). In the present study, Caco-2 cells were found to uptake the nanoparticles to an acceptable extent. Figure 5 demonstrates that 100 nm particles had 2.3-folds greater uptake compared to that of 50nm particles, 1.3-folds to that of 500 nm particles, about 1.8 folds that of 1000 nm particles. Thus, it is demonstrated that nanoparticles of 100 – 200 nm size acquire the best properties for cellular uptake. Not only does this highlight the misconception that the smaller the particle size is, the better the cellular uptake can be resulted, but also bring to light of the fact that there has an optimum size range.

The control experiments performed by incubating Caco-2 cells with medium of the coumarin-6 released from the nanoparticles did not show any significant uptake, which demonstrates that the raw coumarin-6 markers can not be directly internalized by the cells (Fig. 4.5). Furthermore, the uptake of nanoparticles by Caco-2 cells was found increased with increase in the concentration of nanoparticles in the medium. 9.4% of nanoparticles were taken up at the particle concentration of 100 μg/ml, 15.6% at 250 μg/ml and 21.1% at 500 μg/ml (Fig. 4.6).

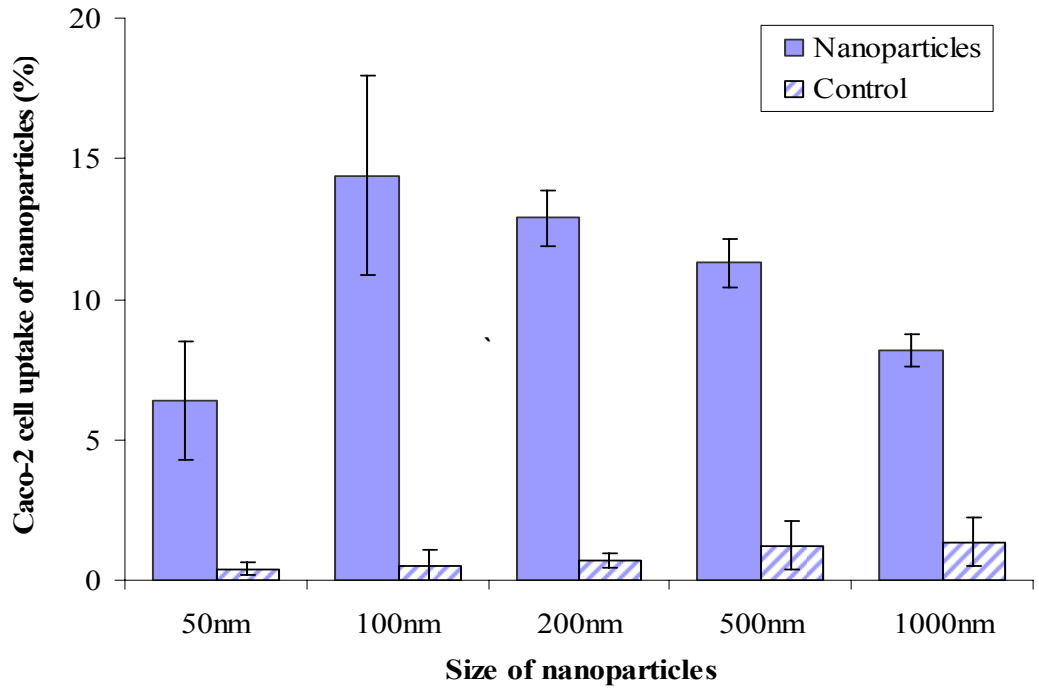


Figure 4. 5. Effect of particle size on cellular uptake by Caco-2 cells of polystyrene nanoparticles after 1 hour incubation at particle concentration of 250 µg/ml at 37°C. The control is the cellular uptake of the coumarin-6 released from the nanoparticles under *in vitro* conditions and incubated with Caco-2 cells at the same conditions. Data represents mean ± SD, n=3.

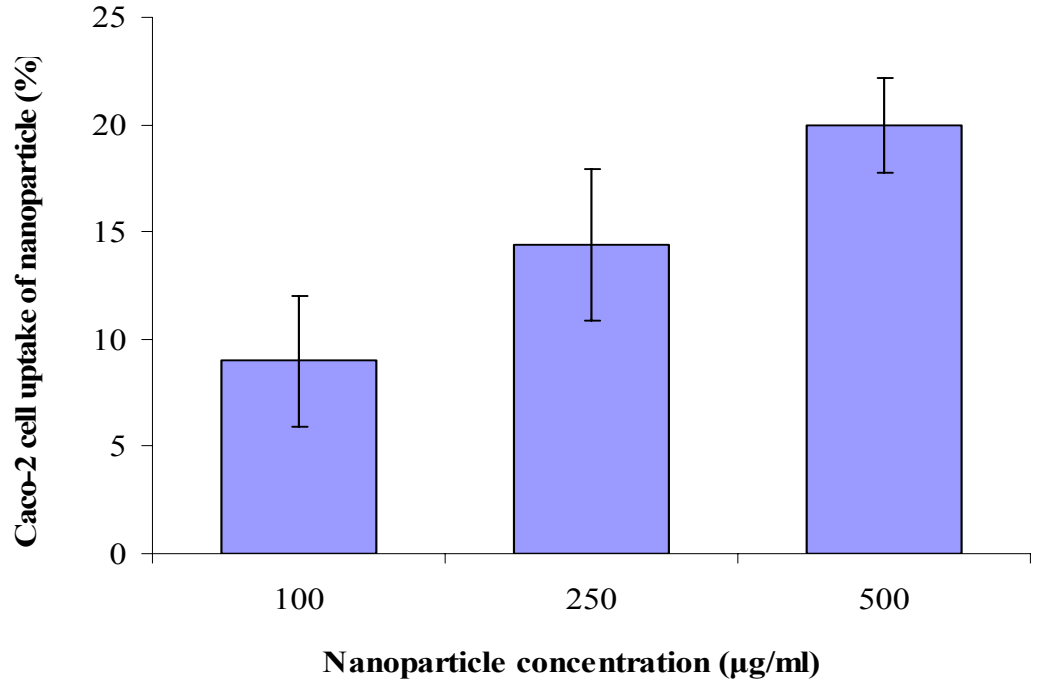


Figure 4. 6. Effect of particle concentration on cellular uptake by Caco-2 cells of 100 nm polystyrene nanoparticles after 1 hour incubation at 37°C. The control is the cellular uptake of the coumarin-6 released from the nanoparticles under *in vitro* conditions and incubated with Caco-2 cells at the same conditions. Data represents mean ± SD, n=3.

Our Caco-2 cell uptake studies exhibit its dependency upon the nanoparticle size with smaller particles possessing greater uptake in general. This supports the findings reported in the literature that the extent of particle uptake is indirectly proportional to the particle size (Desai et al., 1997). The only exception is that the smallest size particles we studied, 50nm, showed the lowest uptake, indicating there may be a limit beyond which the size no longer plays an influencing key role in the extent of uptake. However, due to numerous discrepancies in the literature, no set criteria are available for the design of an appropriate particulate carrier system. A major source of confusion may lie in the large variety of analytical methods and experimental models that have been employed to investigate particle uptake (McClean et al., 1998).

The smaller size particles seem to have efficient interfacial interaction with the cell membrane compared to larger size particles. Probably the larger size particles (> 1μm) are taken up by mechanism other than endocytosis, such as fluid-phase pericytosis (Boudad et al., 2001). Since the small size particles could improve efficacy of the particle-based oral drug delivery systems (Kreuter, 1991; Fujikawa and Kuroda, 2000) and vitamin E TPGS attributes inhibition of P-gp mediated drug transport in addition to increased absorption by enhancing solubility and permeability (Dintaman and Silverman, 1999; Yu et al., 1999), the smaller size nanoparticles with TPGS modified surface could definitely improve the efficiency of cellular uptake and be highly feasible for oral chemotherapy.

4.3.3.3.Confocal microscopy

Figure 4.7 and Figure 4.8 show confocal microscopic images of Caco-2 cell monolayers after nanoparticle uptake experiments, which strongly support the

previous quantitative measurements of the cellular uptake of nanoparticles by showing strong fluorescence in the cell cytoplasm as well as in the nucleus. Effects of particle size, culture time and surface modification on nanoparticle uptake are clearly evidenced. The confocal micrographs in Fig. 4.7 represent the optical sections (x-y axis) of Caco-2 cells after 1 hr incubation with coumarin-6 loaded, PVA-coated (Fig. 4.7a), vitamin E TPGS-coated (Fig. 4.7b), and DPPC-coated (Fig. 4.7c) PLGA nanoparticles. The cells incubated with vitamin E TPGS-coated nanoparticles at 37°C exhibit a thicker layer of stronger fluorescence than those incubated with the PVA-coated nanoparticles under the same other conditions. The cells treated with DPPC-coated nanoparticles demonstrate comparable strong fluorescence intensity, even though slightly weaker, with those TPGS-coated nanoparticles. In these images, a fluorescent layer coinciding with the cell outline can be observed. However, no fluorescence can be detected from the image of the control cells (figure not shown), which were not exposed to the coumarin-6 loaded nanoparticles and/or placebo nanoparticles, implying there is no auto-fluorescence of the cells or polymer material of nanoparticles which can lead to misinterpretation of the data. Three-dimensional analyses of the confocal images of the cells incubated with the vitamin E TPGS-coated and DPPC-coated fluorescent PLGA nanoparticles at 37°C were shown in Fig. 4.8 in which the reconstruction of the z-axis especially clearly demonstrates the fluorescent signals inside the cells. This implicates the actual internalization of the nanoparticles by the Caco-2 cells. In contrast, uptake of polystyrene nanoparticles into Caco-2 cells is very poor (figures not shown). Typically, polystyrene nanoparticles are found attached to the apical cell surface only. No particles could be observed intracellularly or at the basolateral side.

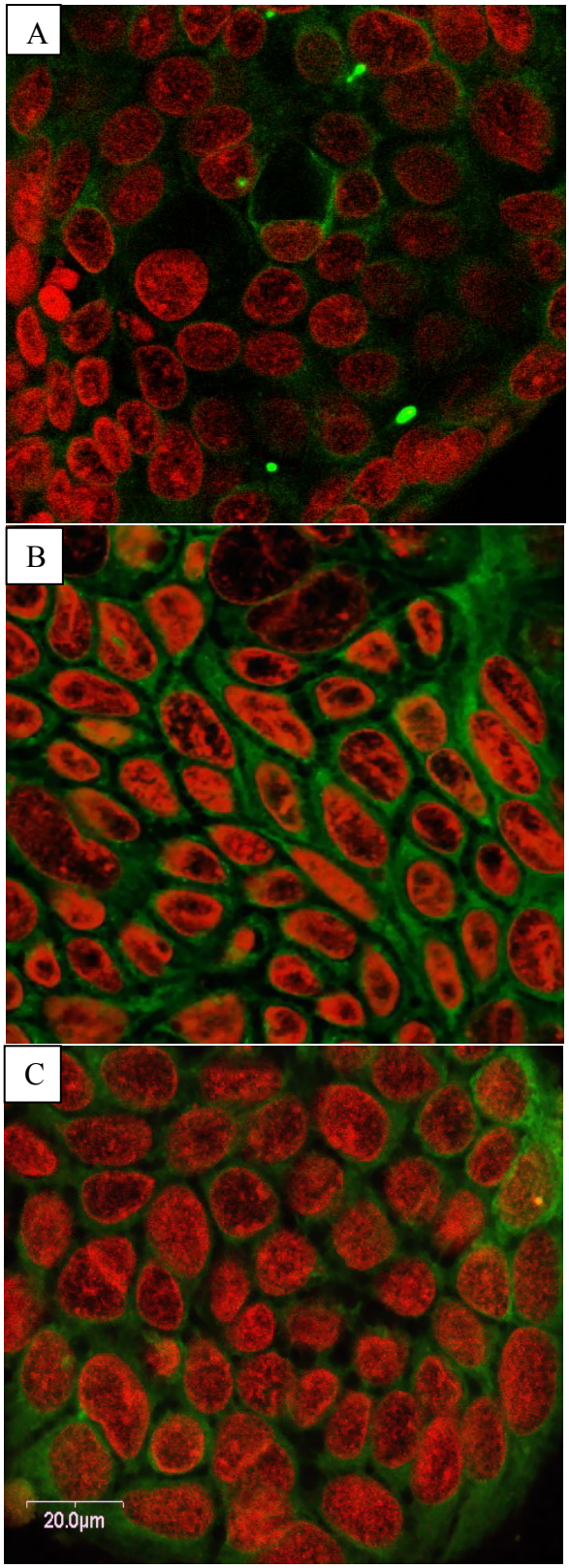


Figure 4. 7. Confocal microscopic images of Caco-2 cells after 1 hour incubation with coumarin 6-loaded PLGA nanoparticles coated with (A) PVA; (B) vitamin E TPGS; and (C)DPPC at 37°C. The cells were stained by propidium iodide (red) and uptake of green fluorescent 6-coumarin-loaded nanoparticles in Caco-2 cells was visualized by overlaying images obtained by FITC filter and RITC filter. These figures show a distinct extent in cellular uptake of the nanoparticles.

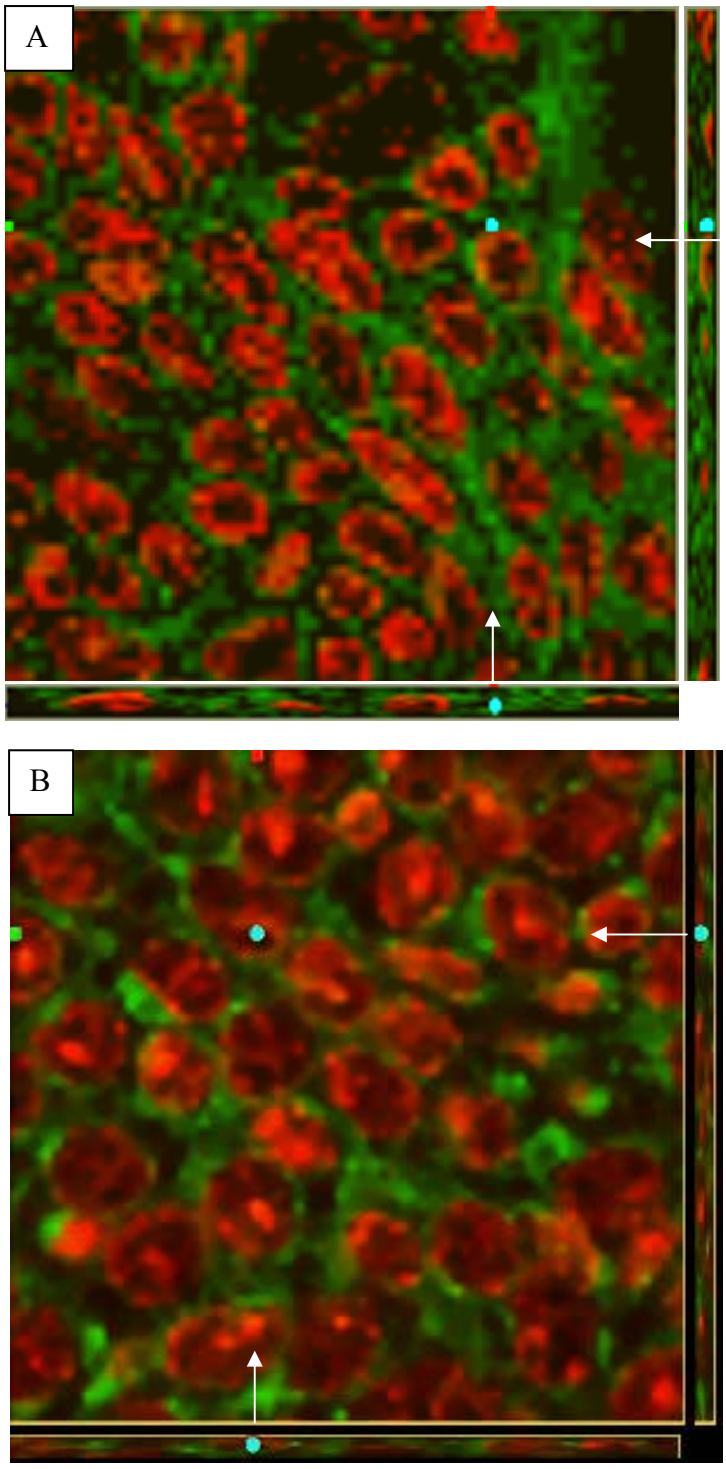


Figure 4. 8. Confocal microscopic images of Caco-2 cells after 1 hour incubation at 37°C with coumarin 6-loaded PLGA nanoparticles coated with vitamin E TPGS (A) and DPPC (B). Optical sections (xy-) with xz- and yz-projections allow to clearly differentiate between the extracellular and the internalised nanoparticles. Small blue circles indicate the plane of section. Green: Fluorescent nanoparticles; Red: Nuclei.

Optical sectioning proved that the nanoparticles were internalized and not simply bound to the surface of the cells (Fig. 4.9 and Fig. 4.10).

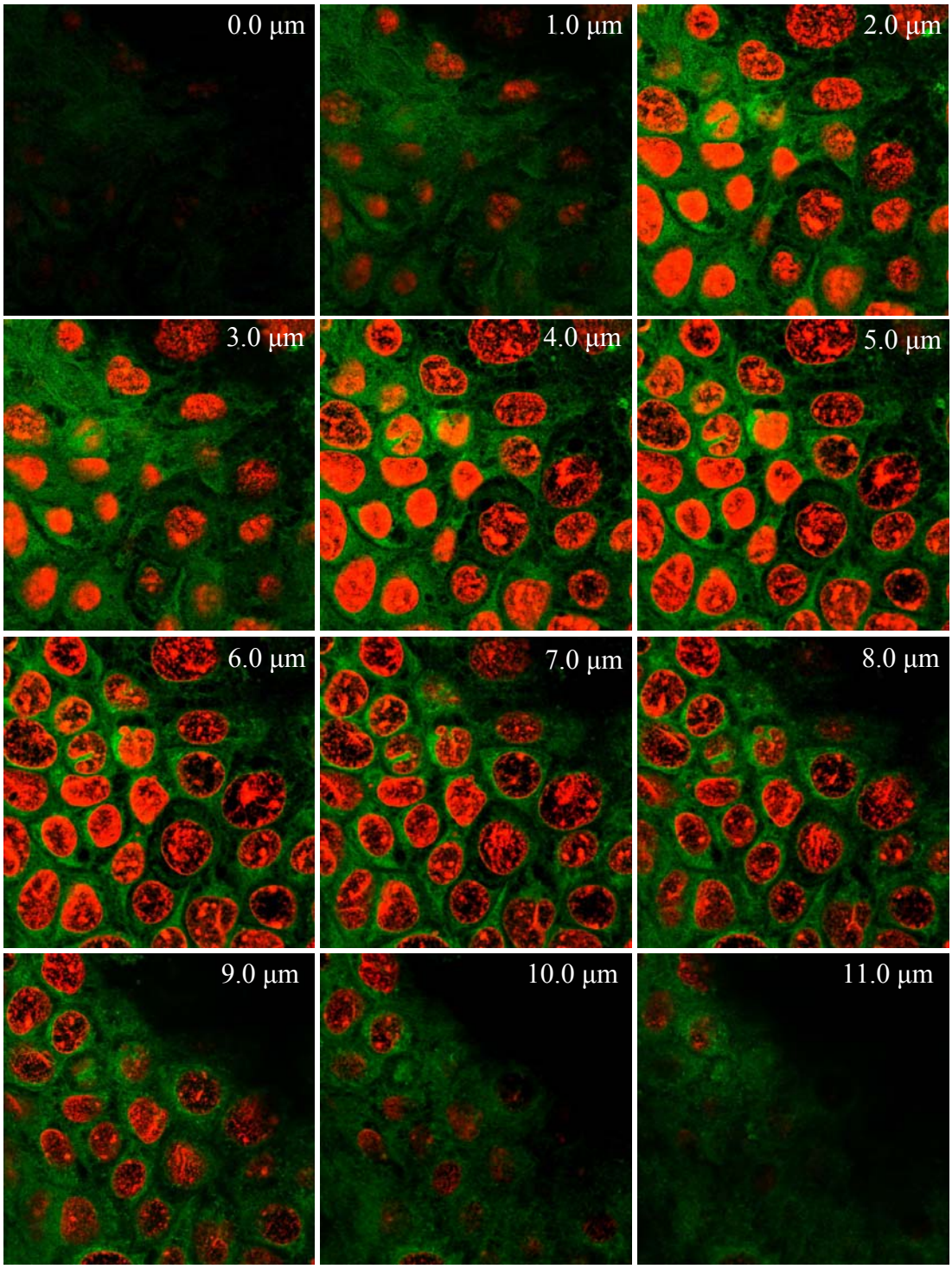


Figure 4. 9. Intracellular distribution of DPPC-coated PLGA nanoparticles in Caco-2 cells after incubated for 1 hr at 37°C as examined by optical sectioning using confocal laser microscope. The focus plane was moved from bottom to top in the vertical axis at an interval of 1.0 µm.

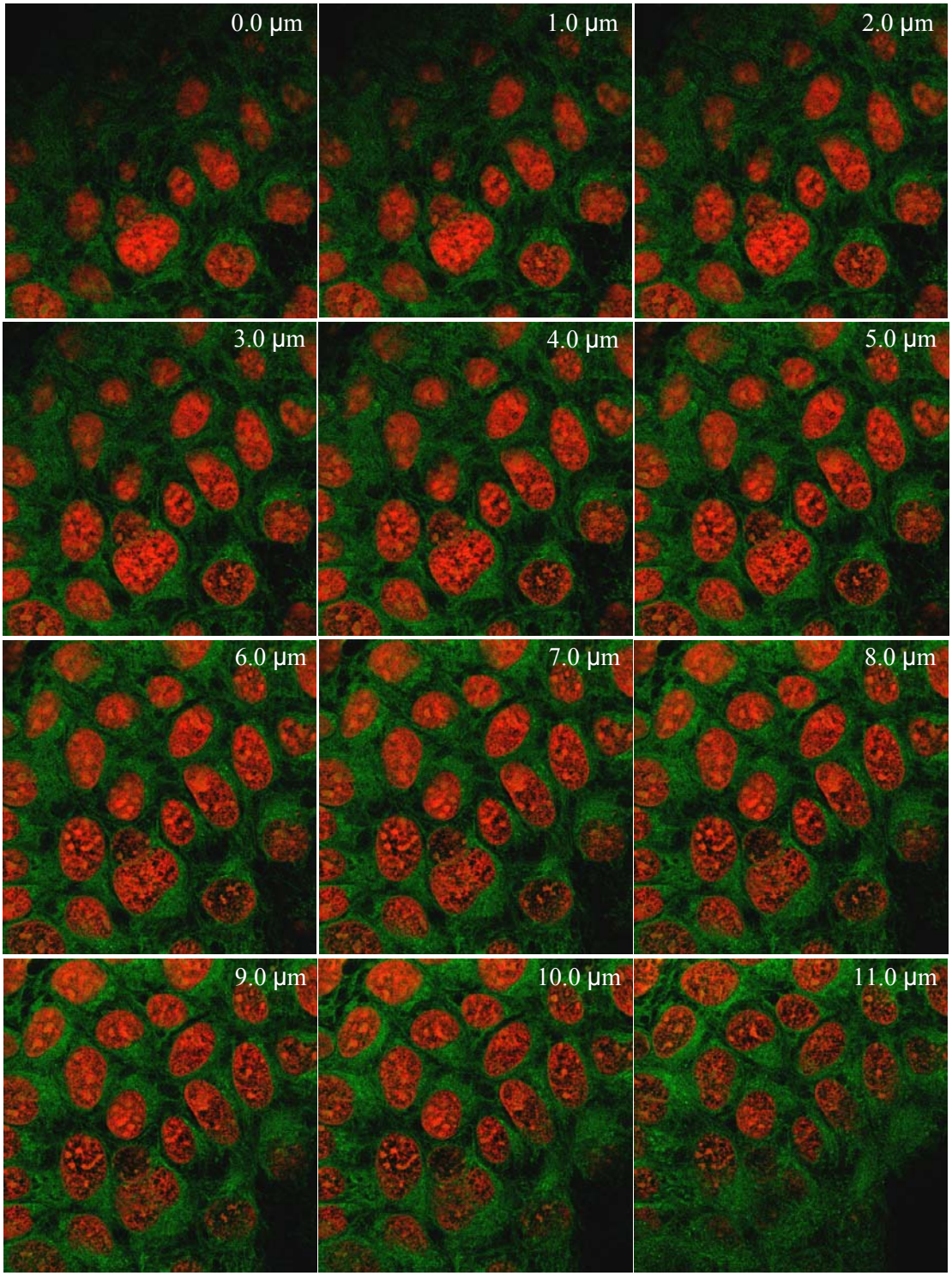


Figure 4. 10. Intracellular distribution of TPGS-coated PLGA nanoparticles in Caco-2 cells after incubated for 1 hr at 37°C as examined by optical sectioning using confocal laser microscope. The focus plane was moved from bottom to top in the vertical axis at an interval of 1.0 µm.

4.3.3.4.Cryo-SEM and TEM

Cryo-SEM enables the observation of bulk biological materials in hydrated conditions by conversion of liquid water to solid by cryo-fixation, which has been widely used for ultrastructural study of biological materials and water distribution within tissues as well as for observation of ice crystal distributions following the freezing of biological materials, especially plant tissues (Fujikawa and Kuroda, 2000). Cryo-SEM can provide direct observation of the cells which are arrested at the existing state with less risk of damaging the cell due to preparation process. Moreover, the preparation of Cryo-SEM samples is much simpler comparing to that of TEM.

Despite the advantages and wide applicable options, there has been no report in the literature on cryo-SEM analysis of cellular uptake of particles to our best knowledge. Figure 4.11 shows the cryo-SEM image of a cross-section of a single Caco-2 cell after treated with vitamin E TPGS-coated PLGA nanoparticles for 1 hr at 37°C, which indeed confirms the efficient uptake and internalization of nanoparticles. The arrows indicate some of the nanoparticles found throughout the endoplasm of the cell and around the nucleus. Some nanoparticles can be found adsorbed on the cell membrane. Some free nanoparticles scattered near the cell can also be observed.

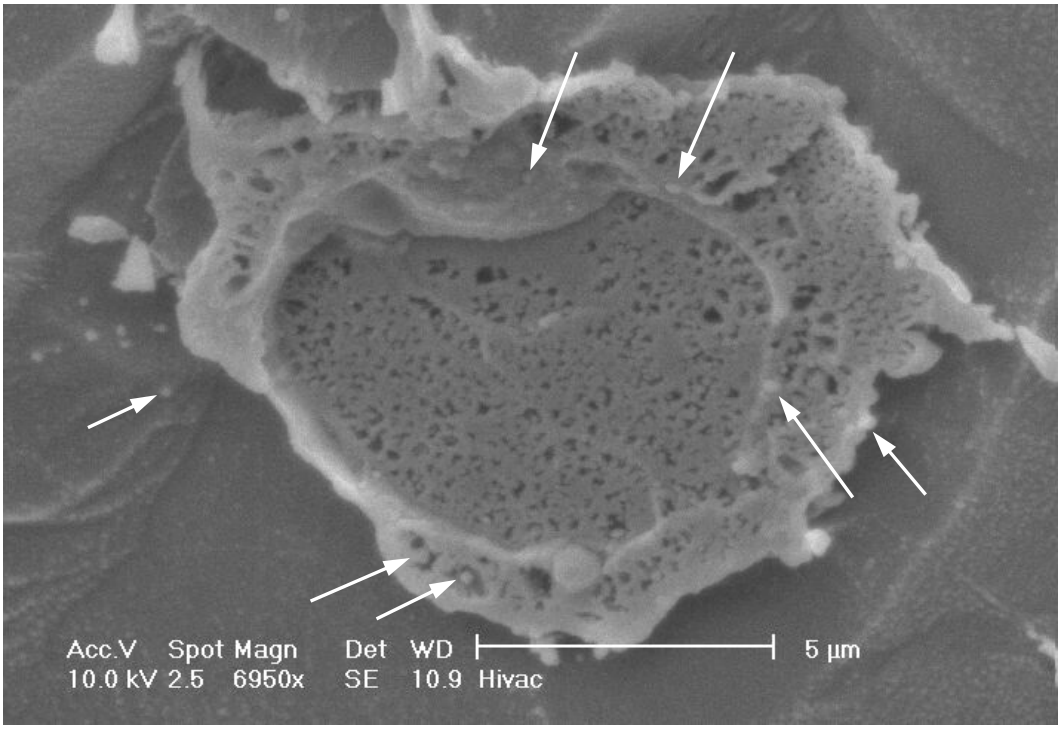


Figure 4. 11. Cryo-SEM image of a cross-section of a typical Caco-2 cell after 1 hour incubation at 37°C with coumarin 6-loaded PLGA nanoparticles coated with vitamin E TPGS. The arrows indicate some nanoparticles found throughout the endoplasm and around the nucleus. Some nanoparticles were found adsorbed on the cell membrane (bar=0.5 um).

Figure 4.12 shows the TEM micrograph of a single Caco-2 cell with uptaken TPGS-coated PLGA nanoparticles of ~200 nm size throughout the cytoplasm and some in the nucleus. The arrows indicate some of the nanoparticles distributed in the cell. The TEM image supports the findings from the cryo-SEM image.

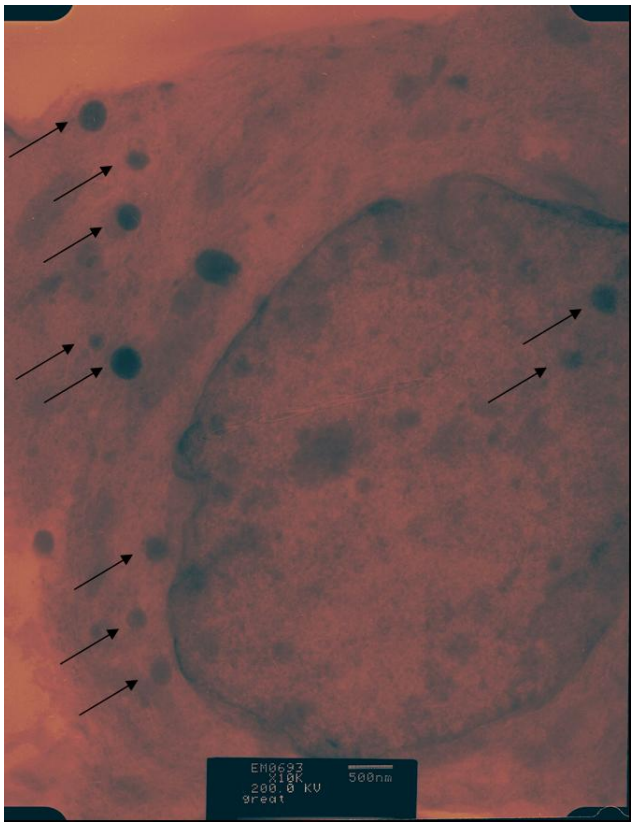


Figure 4. 12. TEM image of a typical Caco-2 cell after 1 hour incubation at 37°C with coumarin 6-loaded PLGA nanoparticles coated with vitamin E TPGS. The arrows indicate some nanoparticles found throughout the endoplasm and within the nucleus (bar=0.5 um).

4.4. Conclusions

Caco-2 cells were used in the present study as an *in vitro* model to evaluate the cellular uptake of fluorescent polystyrene nanoparticles of standard size and coumarin-6 loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles coated with polyvinyl alcohol (PVA) or vitamin E TPGS or DPPC with the aim to promote oral delivery of anticancer drugs by nanoparticles of biodegradable polymers. The emphasis was given to the possible effects of particle size and particle surface coating on the cellular uptake of the nanoparticles. Our results illustrated that PLGA nanoparticles have significantly higher level of the cellular uptake compared with

polystyrene nanoparticles and the vitamin E TPGS coated PLGA nanoparticles have advantages in favor of the cellular uptake over the PVA coated or DPPC coated PLGA nanoparticles. Thus, TPGS coated PLGA nanoparticle formulation was selected for further detailed studies. An observed plateau effect of the cellular uptake efficiency against the nanoparticle concentration or the cell incubation time suggested that the cellular uptake of polymeric nanoparticle is saturable. The internalized nanoparticles were mostly seen throughout the cytoplasm surrounding the nucleus and minority of them were found in the nucleus, especially in the case of vitamin E TPGS coated PLGA nanoparticles, in the images of confocal laser scanning microscopy (CLSM), cryo-SEM and transmission electron microscopy (TEM). Our results demonstrated that nanoparticles of biodegradable polymers of small enough size and with appropriate surface coating may have great potential to be applied for oral delivery of anticancer drugs as well as other therapeutic agents. “Chemotherapy at home” may be a dream which is coming true.

CHAPTER 5

IN VITRO AND *IN VIVO* EVALUATIONS ON PLGA NANOPARTICLES FOR PACLITAXEL FORMULATION

5.1. Introduction

Cancer and cardiovascular diseases are the leading causes of human deaths and cancer is becoming the number 1 killer in many countries including Singapore. However, no substantial progress can be observed in the past 50 years in fighting against cancer. The cancer death rate in US was 1.939% of the total population in 1950 and still 1.940% in 2001. Cancer nanotechnology will change the very foundations of cancer diagnosis, treatment and prevention. The traditional treatment of cancer has been surgery followed by radiotherapy. If the treatment fails, the patients have only 10% chance to be cured by other therapies. Chemotherapy can play more important role and will become one of the most effective treatments available for cancer and other diseases such as cardiovascular diseases and AIDS. Chemotherapy, however, carries a high risk due to the drug toxicity, and the more effective drugs tend to be more toxic. Problems still exist even for successful chemotherapy. The current regimen of chemotherapy is far from being satisfactory. Its efficacy is limited and patients have to suffer from severe side effects. Nanoparticles of biodegradable polymers may provide an ideal solution and promote a new concept of chemotherapy, which may include sustained, controlled and targeted chemotherapy; personalized chemotherapy; chemotherapy across various physiological drug barriers; and eventually, chemotherapy at home (Feng and Chien, 2004; Feng, 2004).

Paclitaxel, a naturally occurring diterpenoid originally extracted from the Pacific Yew tree in the early 1960's, has been considered by the National Cancer Institute (NCI) as the most significant advance in drug discovery for chemotherapy and has become commercially most successful antineoplastic agent. The drug has been known to exhibit a significant activity against a wide spectrum of cancers, including breast cancer, ovarian cancer, colon cancer, bladder cancer, lung cancer, head and neck carcinomas, and acute leukemia (Wani et al., 1971; Spencer and Faulds, 1994; Thigpen, 2000; Ishitobi et al., 2001; Chang et al., 2001; Davidson, 2002). The mechanism of antitumor activity of paclitaxel is unique. It inhibits cellular growth by promoting and stabilizing microtubule assembly by non-covalent interaction with tubulin, thereby blocking cell replication in the late G2 mitotic phase of the cell cycle (Kumar, 1981; Manfredi and Horwitz, 1984; Horwitz, 1994; Huizing et al., 1995). Paclitaxel has several drawbacks including its marked nephrotoxicity and little oral bioavailability. However, the main limitation for its clinical application is its low solubility in water and most of the pharmaceutical solvents (Panchagnula, 1998). Its dosage form Taxol® is formulated in Cremophor EL and dehydrated alcohol at a 50:50 (v/v) ratio, which has been found associated with severe side effects including hypersensitivity reactions, nephrotoxicity, neurotoxicity and cardiotoxicity (Weiss et al., 1990; Waugh et al., 1991; Dorr, 1994; Szebeni et al., 1998; Gelderblom et al., 2001; Singla et al., 2002). Alternative paclitaxel formulation strategies have been suggested to eliminate such an adjuvant and to improve its therapeutic efficacy, which include parenteral emulsions (Tarr et al., 1987; Ludenberg, 1997; Kan et al., 1999), liposomes (Sharma and Straubinger, 1994; AI-Angary et al., 1995; Crosasso et al., 2000), micelles (Liggins and Burt, 2002), nanoparticles (Sharma et al., 1996; Feng et al., 2000; Feng and Huang, 2001; Chen et al., 2001; Kim and Lee, 2001; Feng et al.,

2004) and microspheres (Wang et al., 1996; Sato et al., 1996; Mu and Feng, 2001; Liggins and Burt, 2001; Ruan and Feng, 2003). Among them, nanoparticles of biodegradable polymers could provide an ideal solution as well as realize a sustained, controlled and targeted delivery of the drug and promote oral chemotherapy.

There has been intensive investigation directed to oral delivery of paclitaxel, which is expected to provide a long-time exposure at an appropriate therapeutic level of drug and to greatly improve the quality of life of the patients. The obstacle to the successful formulation of oral dosage form is its low oral bioavailability (less than 1%) due to the elimination by the multi-drug efflux pump transporter P-glycoprotein (P-gp) (Sparreboom et al., 1997) and the first pass of cytochrome P450 enzymes (Cresteil et al., 1994; Harris et al., 1994). Medical solution to overcome this problem is to apply P450/P-gp inhibitors such as cyclosporin A (Scambia et al., 1995; Bardelmeijer et al., 2000; Choi et al., 2004). However, the inhibitors would also fail the immune system of the patients and thus lead to medical complications. Moreover, most of the P450/P-gp inhibitors have their own side effects and difficulties in formulation (Bonduelle et al., 1996). Nanoparticles of biodegradable polymers represent a chemotherapeutic engineering solution or cancer nanotechnology solution.

Nanoparticles of biodegradable polymers for drug formulation and for oral chemotherapy have shown advantages in improving the pharmacokinetics and tissue distribution and thus the therapeutic effects of the formulated drug (Couvreur et al., 1980; Rolland, 1989). Preliminary results have demonstrated that nanoparticles can escape from the vasculature through the leaky endothelial tissue that surrounds the tumor and thus accumulate in solid tumors (Leroux et al., 1996; Monsky et al., 1999). It was demonstrated that nanoparticle formulation can overcome the multi-drug

resistance phenotype mediated by P-glycoprotein and thus lead to an increase in drug content inside the neoplastic cells (Seelig, 1998; Rowinsky and Donehower, 1995; Bennis et al., 1994).

The key factors which determine the performance of the drug-loaded nanoparticles include the particle size and surface coating (Win and Feng, 2005). Modification of nanoparticle surface could be an important strategy to improve the half life of the nanoparticles in plasma and the cellular uptake of the nanoparticles. We have successfully developed a nanoparticle technique to coat nanoparticles of biodegradable polymers by phospholipids, cholesterol and vitamin E TPGS, which showed great advantages over the traditional coating techniques by polyvinyl alcohol (PVA) and poly(ethylene glycol) (PEG) (Feng and Huang, 2001; Win and Feng, 2005). Vitamin E succinated polyethylene glycol 1000 (TPGS) is a water soluble derivative of vitamin E, which has been found to be an excellent emulsifier/solubilizer/absorption enhancer of high emulsification efficiency and cellular adhesion (Mu and Feng, 2002; Mu and Feng, 2003a and 2003b) as well as a Pg-p inhibitor (Bennis et al., 1994; Rowinsky and Donehower, 1995; Seelig, 1998). Co-administration of vitamin E TPGS has been found to increase the oral bioavailability of cyclosporine A in healthy dogs by non-compartmental pharmacokinetic analysis (Fischer et al., 2002). Dintaman and Silverman (1999) reported vitamin E TPGS inhibits P-glycoprotein mediated multidrug resistance. Vitamin E TPGS can also enhance the absorption flux of amprenavir, a HIV protease inhibitor, by increasing its solubility and permeability (Yu et al., 1999).

The objective of this study was to develop a polymeric drug delivery system for an alternative formulation as well as for oral delivery of paclitaxel, which is used in our

research as a prototype anticancer drug due to its excellent efficiency against a wide spectrum of cancers and its great commercial success as the best seller among antineoplastic agents, In our nanoparticle technique, vitamin E TPGS is used as a necessary auxiliary in nanoparticle formulation as well as a "mask" for the nanoparticles to cross the GI barrier for oral chemotherapy. Paclitaxel-loaded, TPGS-emulsified PLGA nanoparticles were prepared by a modified solvent extraction/evaporation single emulsion technique. Nanoparticles of various recipes were characterized by various state-of-the-art techniques such as laser light scattering for particle size and size distribution, scanning electron microscopy (SEM) for surface morphology, X-ray photoelectron spectroscopy (XPS) for surface chemistry, and high performance liquid chromatography (HPLC) for in vitro drug release kinetics. Caco-2 cells were employed as an established in vitro model of the GI barrier (Artusson, 1990; Desai et al., 1997; Delie, 1998). Human colon adenocarcinoma cells (HT-29 cells) were used to evaluate the cytotoxicity of the drug formulated in the nanoparticles. In vitro and in vivo pharmacokinetics of the drug in nanoparticle dosage form were measured and analyzed in a close comparison with its current clinical dosage form, Taxol®.

5.2. Materials and methods

5.2.1. Materials

Paclitaxel of purity 99.8% was purchased from Dabur India Ltd. (India). The details of the materials were the same as reported in section 3.2.1 and 4.2.1. All chemicals were of the highest grade available commercially.

5.2.2. Nanoparticle preparation

Nanoparticles with drug or coumarin-6 were prepared by a modified solvent extraction/evaporation single emulsion technique as mentioned in section 3.2.2. In brief, an oil phase solution of DCM containing PLGA (50:50) and paclitaxel was slowly poured into an aqueous solution containing PVA or vitamin E TPGS and emulsified using a microtip probe sonicator at an energy output of 20Watts in pulse mode. For fluorescent nanoparticles, the drug was replaced by 0.05% (w/v) coumarin-6 as a fluorescent marker. The resultant oil-in-water emulsion was then stirred at room temperature by a magnetic stirrer to evaporate DCM. The produced nanoparticles were collected by centrifugation and washed with Millipore water for three times to remove excessive emulsifier and drug or fluorophore. The nanoparticle suspension was then freeze-dried to obtain fine powder of nanoparticles, which was kept in a vacuum desiccator. The theoretical loading ratio of paclitaxel in the nanoparticles was set to be 5%. Formulation optimization was pursued to meet various therapeutic needs.

5.2.3. Characterization of nanoparticles

5.2.3.1. Size and size distribution

The size and size distribution of the formulated nanoparticles were determined by laser light scattering with particle size analyzer (90 Plus, Brookhaven Inst, Huntsville, NY) at a fixed angle of 90° and 25°C. The data reported were obtained from the average of 5 measurements.

5.2.3.2. Morphology of nanoparticles

The morphology of nanoparticles was observed by scanning electron microscopy (SEM). SEM (JSM 5600LV, Jeol, Tokyo) requires an ion coating with platinum by a sputter coater (JFC-1300, Jeol, Tokyo) for 40 seconds in a vacuum at a current intensity of 40 mA after preparing the sample on metal studs with double-sided conductive tape. The accelerating voltage ranged from 10-15 kV during scanning. The size of the formulated nanoparticles was confirmed by measuring the diameters in the SEM images by using the SmileView software.

5.2.3.3. Surface properties of nanoparticles

Zeta potential is an indicator of surface charge, which determines particle stability in dispersion. The zeta potential of the formulated nanoparticles was determined by a zeta potential analyzer (Zeta Plus, Brookhaven Instruments, Huntsville, NY) by dipping the palladium electrode in the sonicated particle suspension of interest and the means of 10 readings were reported.

Surface chemistry of nanoparticles was analyzed by X-ray photoelectron spectroscopy (XPS, AXIS His-165, Kratos Analytical, Shimadzu). For all samples, the angle of X-ray used was 90°C and the survey spectrum recorded covered a binding energy range from 0 to 1200 eV with pass energy of 80 eV. Curve fitting was performed using the software supplied by the manufacturer.

5.2.3.4. Drug encapsulation efficiency

The amount of drug encapsulated in the nanoparticles was determined in triplicates by high performance liquid chromatograph (HPLC, Agilent LC 1100 series). 3 mg of nanoparticles was dissolved in 1 ml of DCM and 3 ml of acetonitrile-water (50:50) was then added. A nitrogen stream was introduced to evaporate the DCM until a clear solution was obtained. The solution was put into vials to detect the paclitaxel concentration by HPLC analysis using a reverse phase Inertsil® ODS-3 column. 50 µl of sample was injected by an auto-sampler and the column effluent was detected at 227 nm with a variable wavelength detector. The calibration curve was prepared for the quantification of drug in the nanoparticles and it was linear over the range of 50-10,000ng/ml with a correlation factor of $r^2 = 0.9999$.

To correct the error arising from possible inefficient extraction, the recovery efficiency factor of the extraction procedure was determined. The resulted recovery was 91.99 %, which implies that 91.99 % of the original amount of paclitaxel could be extracted in the process and thus all the data obtained should be corrected accordingly to determine the drug encapsulation efficiency in the formulated nanoparticles. The encapsulation efficiency of paclitaxel was obtained as the mass ratio between entrapped amount of paclitaxel from the formulated nanoparticles and that added in the preparation.

5.2.4. *In vitro* drug release

The drug-loaded nanoparticles were dispersed in a simulated physiological fluid, PBS, at pH 7.4. The nanoparticle suspension was kept in an orbital shaker at constant gentle shaking of 110 rpm at 37°C. At pre-determined time intervals, the suspensions were centrifuged at 10,000 rpm at 37°C for 15 min. The precipitated particles were re-suspended in fresh buffer and placed back into the shaker. The released paclitaxel in the supernatant was first extracted with 1 ml of DCM, followed by 3 ml of the mixture of acetonitrile and water (50:50, v/v), then evaporated under a stream of nitrogen until a clear solution was obtained. HPLC analysis was then conducted as previously described.

Due to possible inefficient extraction process of the highly hydrophobic paclitaxel, the extraction factor had to be determined by measuring the extracted amount of drug for a known amount of paclitaxel which underwent the same extraction procedure. It was found that only 68 % of the original paclitaxel could be extracted by the procedure. Therefore, all the data obtained from *in vitro* release studies were corrected accordingly.

5.2.5. Cell Culture

In this study, Caco-2 cells (ATCC, VA) of passages between 26 and 31 were maintained in MEM medium supplemented with 20% FBS, 100mM sodium pyruvate, 1.5g/L of sodium bicarbonate and 1% penicillin-streptomycin solution. Human colon adenocarcinoma cells (Caco-2), HT-29 cells (ATCC, VA) of passages between 20 and 24 were cultured in McCoy's 5A medium with 1.5mM L-glutamine, supplemented

with 10% FBS, 2.2g/L of sodium bicarbonate and 1% penicillin-streptomycin solution. Cells were seeded at 4.3×10^4 cells/cm^2 in the 96-well black plates with transparent bottom (Costar, IL, USA) for quantitative uptake experiments or on coverglass in chamber system for confocal microscopy, and in the 96-well transparent plates (Costar, IL, USA) for cytotoxicity experiments. Cells were cultured as a monolayer at 37°C in a humidified atmosphere containing 5% CO_2. Medium was replenished every other day.

5.2.6. *In Vitro* Cellular Uptake of Nanoparticles

To measure the cellular uptake of nanoparticles, Caco-2 cells were seeded in 96-well black plates (Costar, IL, USA) and incubated until they formed a confluent monolayer. Upon reaching confluence, the culture medium was replaced by transport buffer (HBSS, pH 7.4) and pre-incubated at 37ºC for 30 min. After equilibration, cellular uptake of nanoparticles was initiated by exchanging the transport medium with 100 µL of the specified nanoparticle suspension (250µg/ml in HBSS). The cells were further incubated for 2 hrs. The experiment was terminated by washing the cell monolayer three times with PBS (pH 7.4) to eliminate the excess nanoparticles, which were not associated to the cells. The cell membrane was permeabilized with 0.5% Triton X-100 in 0.2M NaOH solution to expose the internalized nanoparticles for the measurement. Cell-associated nanoparticle was quantified by analyzing the cell lysate in a fluorescence plate reader (Genios, Tecan, Männedorf, Switzerland, λ_{ex} = 430 nm, λ_{em} = 485 nm). Cellular uptake of the nanoparticles was expressed as the percentage of fluorescence associated with the cells over the total amount of fluorescence in the feed solution.

5.2.7. Confocal laser scanning microscopy (CLSM)

Caco-2 cells were seeded on Lab-Tek® chambered cover glasses (Nalge, Nunc International, Naperville, IL, USA) and incubated at 37°C in 95% air/5% CO_2 environment until cells were about 70% confluent. On the day of experiment, the growth medium was replaced by transport buffer (HBSS, pH 7.4). After equilibration with HBSS at 37ºC for 30 min, the buffer was replaced with the nanoparticle suspension (250µg/ml in HBSS). The monolayers were further incubated for 1 hr. At the end of experiment, the monolayers were washed 3 times with fresh pre-warmed transport buffer to eliminate the excess nanoparticles not taken up by the cells. The cells were then fixed and the cell nuclei were stained by propidium iodide (PI). The samples were mounted in the fluorescent mounting medium (Dako, CA) until examination was performed by the confocal laser scanning microscope (Zeiss LSM 410, Germany) equipped with an imaging software, Fluoview FV300.

5.2.8. *In vitro* cytotoxicity

HT-29 cells were used for cytotoxicity evaluation of the drug formulated in the nanoparticles against that formulated in Cremophor EL (Taxol®) with the drug concentration ranging from 0.25 to 25µg/ml for 24, 48, 72 and 96 hour incubation. Cytotoxicity was determined by MTT assay, in which the optical density at 570 nm was determined by a microplate reader (Genios, Tecan, Männedorf, Switzerland). The amount of the formazan product which is determined by absorbance measurement is directly proportional to the number of viable cells. This is because only the viable cells interact with MTT to form the purple blue formazan precipitates.

5.2.9. Detection of internucleosomal fragmentation

HT-29 cells were seeded at a density of $1x10^5$ cells/chamber on Lab-Tek® chambered cover glass system and incubated at 37°C. Upon reaching about 70% confluency, cells were incubated with the paclitaxel loaded nanoparticles of interest and Taxol® which were adjusted with the culture medium to have the same paclitaxel concentration of 2.5μg/ml. for a predetermined time. Cells incubated with the culture medium served as the control. Experiment was terminated by removing the paclitaxel formulations and washing with PBS for 3 times and fixing the cells with 70% ethanol solution for 20min. The cells were washed with PBS for 2 times and stained with PI (20μg/ml) for 20min. Excess PI was removed by washing with PBS for 3 times and cells were mounted in Dako fluorescent mounting medium until confocal microscopy.

5.2.10. *In vivo* pharmacokinetics

Care and handling of animals, and animal experiment protocols were approved by the Institutional Animal Care and Use Committees (IACUC), Office of Life Science, National University of Singapore. The in vivo pharmacokinetics was measured with male Sprague-Dawley rats of 180-200gm and 6-8 week old, which were supplied by the Laboratory Animals Centre of Singapore and were maintained at the Animal Holding Unit of National University of Singapore. The animals were housed in air-conditioned facility and provided with standard food and filtered water. Animals were randomly assigned to three groups of three rats. Group 1 received an i.v. injection and Group 2 received an i.v. infusion of paclitaxel-loaded TPGS-emulsified PLGA nanoparticles. Group 3 received an i.v. injection of Taxol®. The nanoparticles were dispersed with saline for dosing at a concentration of 10mg/ml. Taxol® was diluted

with saline for dosing at a paclitaxel concentration of 1mg/ml. The formulations were administrated through the tail vein at the paclitaxel concentration of 10mg/kg of the rat body weight. All animals were observed for mortality, general condition and potential clinical signs.

For Groups 1 and 2, blood samples were collected at 0 (pre-dose), 0.5, 1, 3, 5, 10, 24, 48 hrs post-treatment. For Group 3, blood samples were collected at 0 (pre-dose), 0.5, 1, 2, 3, 5, 8, 12 and 24 hrs after administration of the drug. Plasma samples were harvested by centrifugation at 1500 × g for 10 min and stored at -20°C until analysis. Liquid-liquid extraction was performed prior to analysis by LC/MS/MS. Briefly, 5µl of internal standard (2µl/ml, in methanol) was added to 50µl of rat plasma and the mixture was extracted with 1ml of tert-butyl methyl ether on a vortex-mixer for 30 s. Upon centrifugation at 2000 × g for 15 minutes, the organic layer was transferred to a clean tube and evaporated under nitrogen at room temperature. The residue was reconstituted with 100µL of mobile phase (acetonitrile:H_2O = 60:40 v/v) and transferred to autosampler vials containing limited-volume inserts (100µL) before analyzed by LC/MS/MS (Wang et al., 2003). Pharmacokinetic parameters were expressed as area under the curve (AUC) and sustainable therapy index which is determined by the ratio of the time the plasma paclitaxel concentration of Taxol reached to the minimum effective level to that of the nanoparticle formulation administrated animal.

5.3. Results and discussions

5.3.1. Size, surface morphology and zeta-potential of nanoparticles

The physicochemical characteristics of the formulated nanoparticles such as particle size and size distribution, zeta potential and drug encapsulation efficiency were summarized in Table 5.1. It can be seen that TPGS has great advantages over PVA in the emulsification process. The emulsification efficiency of TPGS is 66.7 times higher that that of PVA, which means that the amount of TPGS needed to formulate the drug-loaded nanoparticles is only 1/66.7 of that of PVA to formulate the same amount of the nanoparticles. TPGS emulsified NPs (polydispersity = 0.012) are much more uniform in size than the PVA-emulsified NPs (polydispersity = 0.110). The nanoparticles were obtained in the size range of 200-400 nm. The size and size distribution of the drug-loaded nanoparticles are crucial parameters in the design of a particulate drug delivery system since they are main factors in determining the drug release kinetics and cellular uptake efficiency of the nanoparticles (Rome et al., 1994). Moreover, the particle size is associated with the biological response of the tissue to foreign biomaterials (Anderson, 1997; Labhasetwar et al., 1998). Nanoparticles of FDA approved biodegradable polymers usually cause little or no focal inflammation. The inflammatory reactions ensuing fibrosis is a typical response of vascular tissue to microparticles ranging in size between 5 and 10μm (Gradus-Pizlo et al., 1995; Dev et al., 1997). Thus, nanoparticle formulation was effectively attuned to achieve the desired size which falls within a suitable size range (200-500nm) for drug delivery as well as for oral administration of drugs (Couvreur and Puisicux, 1993; Win and Feng, 2005).

Drug encapsulation efficiency plays a key role in formulation of a drug delivery system especially for expensive drugs and directly related to the therapeutic effects of the system. The encapsulation efficiency of paclitaxel in our nanoparticles formulation was 65 – 90% which is quite satisfactory in comparison with other work in the literature.

Table 5. 1. Physicochemical characteristics of paclitaxel-loaded PLGA nanoparticles, fluorescent PLGA nanoparticles and standard PS nanoparticles

Sample	Emulsifier (w/v)	Size ± SD (nm)	Polydis-persity	ζ (mV)	EE (%)
PVA NPs	2 % PVA	294 ± 4.8	0.110	-26.0	89
TPGS NPs	0.03 % TPGS	236 ± 15	0.012	-35.6	66
PVA FL NPs[#]	2 % PVA	243 ± 8.7	0.173	-29.4	-
TPGS FL NPs[#]	0.03 % TPGS	283 ± 4.0	0.140	-31.2	-
200nm FL NPs[*]	-	201 ± 2.3	0.036	-36.8	-
500nm FL NPs[*]	-	498 ± 8.4	0.136	-29.2	-
1000nm FL NPs[*]	-	1007 ± 35	0.125	-27.5	-

[#] Fluorescent PLGA nanoparticles
[*] Standard fluorescent Polystyrene nanoparticles

Surface morphology of the formulated PLGA nanoparticles and fluorescent polystyrene nanoparticles can be observed by scanning electron microscopy (SEM, Fig. 5.1). The images revealed that formulated nanoparticles have regular spherical shape with narrow size distribution and smooth surface without any noticeable pinholes, tiny pores or cracks at the SEM resolution level. No apparent aggregation was detected.

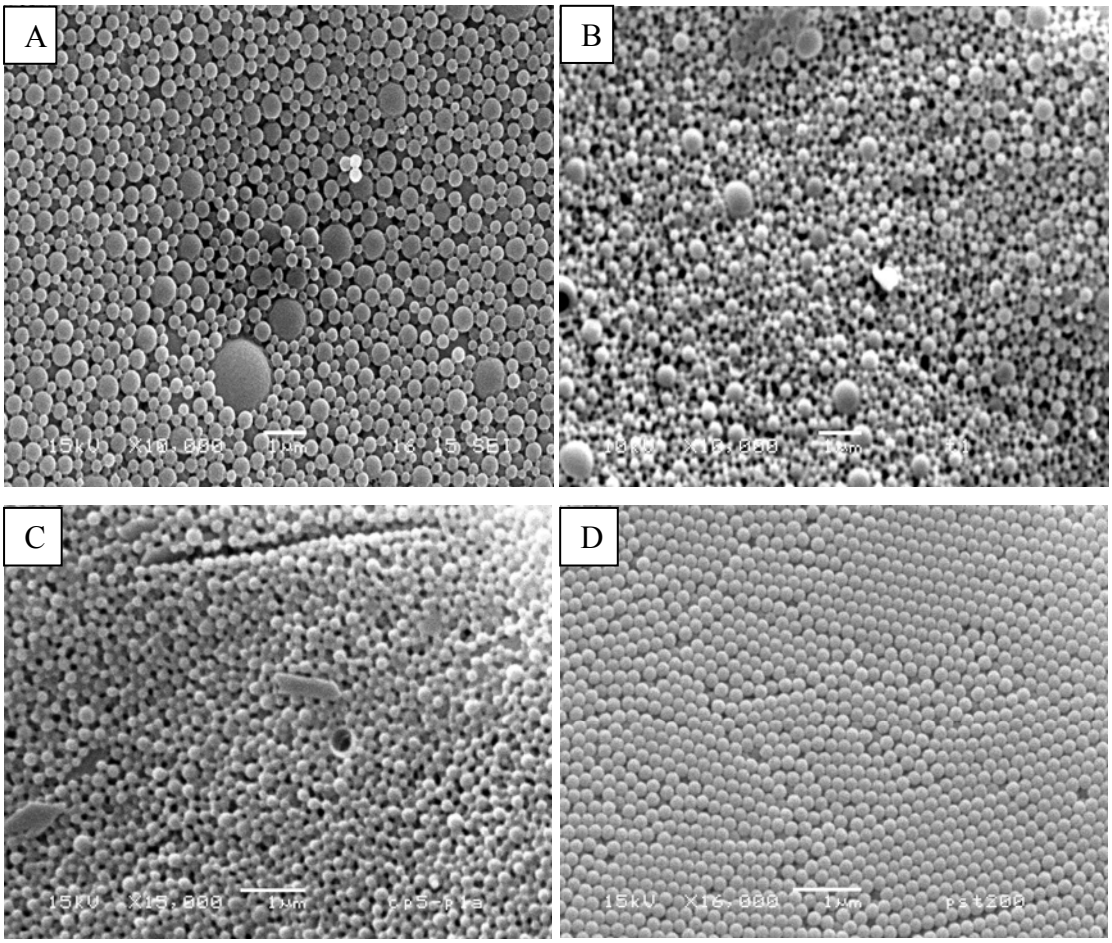


Figure 5. 1. SEM images of paclitaxel-loaded PLGA nanoparticles emulsified by PVA (A) and vitamin E TPGS (B); coumarin-6-loaded PLGA nanoparticles emulsified with TPGS (C); and 200nm fluorescent polystyrene nanoparticles (D) (bar = 1 µm).

Zeta potential of the formulated nanoparticles was found to be strongly influenced by the emulsifier applied in fabrication of the nanoparticles. Although both formulations of the PVA and the TPGS emulsified nanoparticles exhibited negative surface charge (Table 5.1), the latter possessed higher absolute value of zeta potential (-35.60 mV) than the former (-26.05 mV), indicating more stable dispersion can be formed for the TPGS-coated nanoparticles.

5.3.2. Surface chemistry of nanoparticles

It is of interest to analyze the surface chemistry of the formulated nanoparticles, which was carried out by the X-ray photoelectron spectroscopy (XPS). The results are shown in Table 5.2. From the chemical structures of PLGA, PVA, vitamin E TPGS and paclitaxel, it is apparent that paclitaxel is the only substance which contains nitrogen. Thus, nitrogen can be the characteristic element of paclitaxel on the surface of the formulated nanoparticles. Since the scan of nitrogen failed to detect the existence of N1s (atomic orbital 1s of nitrogen) core-level signal on the exterior of the nanoparticles, it is clear that there was no or little (if any) paclitaxel on the surface of the drug-loaded PLGA nanoparticles. This fact implicated that the drug tends to stay inside the polymeric nanoparticles but not on the particle surface as it is highly hydrophobic. The XPS C1s (atomic orbital 1s of carbon) regions were then studied for the pure PLGA, PVA and TPGS as well as the paclitaxel-loaded PLGA nanoparticles emulsified with PVA and TPGS, respectively. The existence of different carbons varies according to the corresponding chemical structures and the amount of the emulsifier on the surface since the depth XPS can detect is limited to 50Å. Table 5.2 shows the quantification of each carbon environment of the polymer, the pure emulsifiers and the formulated nanoparticles in summary. PLGA expresses carbon in C-C or C-H, carbon of ether and carbon of carboxylate and percentage of these 3 carbons vary little from one to another since the contribution of these 3 carbons is close in the molecular chain of PLGA but does not acquire carbon of ether while pure PVA and TPGS attain carbon of ether but lack carbon of ester. In comparison of the XPS peaks of the paclitaxel-loaded, PVA- or TPGS-emulsified PLGA nanoparticles with those of pure PVA, TPGS, and PLGA respectively, it was evident that incorporation of PVA or TPGS provides surface coating or co-existence on the

surface of the nanoparticles. The presence of all four carbons including C-O-C ether peak in the C1s spectrum of PVA or TPGS incorporated PLGA nanoparticles confirms the presence of PVA or TPGS on the surface of nanoparticles since there is no such peak in pure PLGA. Furthermore, the atomic composition of these carbons falls around the values determined for pure PLGA and PVA and/or TPGS which strongly suggests a co-existence of both PLGA and PVA and/or TPGS on the surface.

Table 5. 2. Surface chemistry of the formulation materials and the paclitaxel-loaded PLGA nanoparticles

Sample	XPS C1s envelope ratios (%)			
	C-C	C-O-C	C-O-C=O	O-C=O
PLGA	33.81	-	33.29	32.90
PVA	48.72	46.67	-	4.81
TPGS	53.40	33.81	-	12.79
PLGA/PVA NPs	57.33	24.88	2.26	15.53
PLGA/TPGS NPs	53.95	25.87	2.39	17.79

5.3.3. *In Vitro* drug release

The *in vitro* release profiles of paclitaxel from the PLGA nanoparticles with drug loading 5% (w/w) are shown in Fig. 5.2, from which the effect of surface coating of the nanoparticles on the *in vitro* drug release behavior can be observed. The drug release kinetics exhibited a biphasic pattern characterized by a fast initial burst during the first 24 h, followed by a slow, sustained release. After 30 days, the accumulated drug release was found to be 33% and 28% of the entrapped amount in the vitamin E TPGS-emulsified and the PVA-emulsified PLGA nanoparticles, respectively. Vitamin

E TPGS coating obviously enhanced the drug release rate compared with the conventional PVA-coated nanoparticles. The reason could be that TPGS acts to solubilize lipophilic paclitaxel and thus facilitates its release from the nanoparticles.

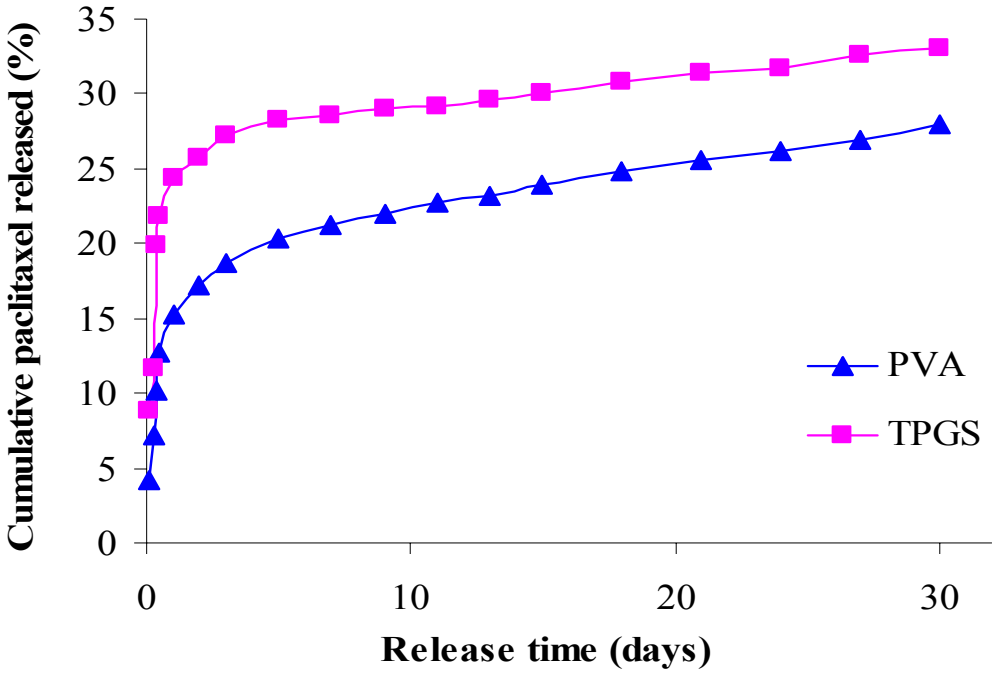


Figure 5. 2. *In vitro* drug release profiles of paclitaxel-loaded PLGA nanoparticles emulsified by PVA and vitamin E TPGS, respectively. Each point represents the mean with ± standard deviation obtained from triplicates of the samples.

The drug release profile plays important role in determining the therapeutic effects of the drug delivery system. It is believed that the therapeutic effect of a dosage form is determined by the area-under-the-curve (AUC) of the time course of the *in vivo* concentration of the drug released from the nanoparticles. The drug release kinetics can be controlled by varying the composition and process parameters such as the polymer type, its molecular weight and copolymer blend ratio, the type and amount of emulsifier, the drug loading ratio, the mechanical strength of mixing, the pH and temperature (Mu and Feng, 2003b). One of the advantages of nanoparticle

formulation is that any desired pharmacokinetics can always be achieved by a specific design to meet the needs of individual patients.

5.3.4. *In vitro* cellular uptake of nanoparticles

Nanoparticles for oral chemotherapy need to be able to cross the gastrointestinal (GI) barrier. The GI barrier is believed to be caused by the tight junctions of intestinal endothelial cells, which are also rich in the multi-drug pump p-glycoproteins (Malingre et al., 2001; Malingre et al., 2000a; Malingre et al., 2000b). The colon adenocarcinoma (Caco-2) cell line was used in this study to simulate the GI barrier since confluent Caco-2 monolayers form tight junctional complex, exhibit dome formation, and possess electrical properties similar to those of the intestinal epithelium. The results are shown in Fig. 5.3 and 5.4.

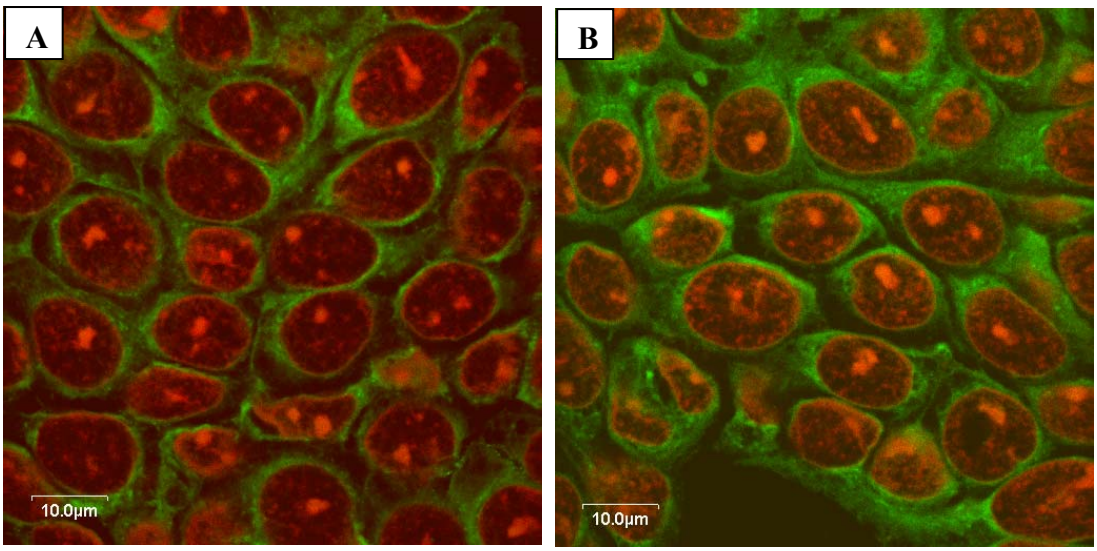


Figure 5. 3. Confocal microscopic images of Caco-2 cells after 1 hour incubation with coumarin 6-loaded PLGA nanoparticles emulsified by (A) PVA or (B) vitamin E TPGS at 37°C. The cells were stained by propidium iodide (red). The uptake of green fluorescent Coumarin 6-loaded nanoparticles in Caco-2 cells was visualized by overlaying images obtained by green filter and red filter. The two figures show a distinct extent in cellular uptake of the nanoparticles depending on their surface coatings.

Cellular uptake of nanoparticle was evidenced by the confocal microscopy of the cell monolayers. Figure 5.3 represents the optical sections (x-y axis) of Caco-2 cells after 1 hr incubation with coumarin-loaded, PVA- or vitamin E TPGS-coated nanoparticles. Cells incubated with the vitamin E TPGS-coated nanoparticles at 37°C (Fig. 5.3b) exhibited a thicker layer of stronger fluorescence intensity than those incubated with the PVA-coated nanoparticles under the same conditions (Fig. 5.3a). In both images, a fluorescent layer coincident with the cell outline was observed. However, there was no fluorescence detected in the control cells (figure not shown) that were not exposed to the coumarin 6-loaded nanoparticles, implying there was no auto-fluorescence of the cells which can lead to misinterpretation of the data. We hypothesized that TPGS could be absorbed intact readily in the GI tract and could inhibit P-gp recognition to enhance nanoparticle absorption to the cells (Dintaman JM, Silverman JA. 1999; Mu and Feng, 2002).

To investigate the particle size effect, commercially available fluorescent polystyrene (PS) nanoparticles of uniform size were employed in the present study. The uptake amount of the PS nanoparticles was found 2.7-folds higher when size was reduced from 1,000nm to 200nm (Fig. 5.4). However, size effect was found not pronounced between the 200 nm and 500 nm standard fluorescent PS nanoparticle. Thus, 200 – 500 nm can be regarded as the ideal size range of nanoparticles for oral drug delivery system.

Surface coating is another important factor to determine the cellular uptake of the nanoparticles. Figure 5.4 shows that the uptake of the drug-loaded PLGA nanoparticles by Caco-2 cells is significantly higher for the TPGS-emulsified nanoparticles than that for the PVA-emulsified nanoparticles. Also, vitamin E TPGS

coating on the drug-loaded PLGA nanoparticles improved the cellular uptake by 1.6-folds over that of the PVA-coated PLGA nanoparticles, 5.5-folds over 200nm PS nanoparticles (with no coating) though they possessed about the same particle size, 6.5-folds over the 500nm PS particles, and 14.6-folds over the 1,000nm PS particles. PVA-coated PLGA nanoparticles enhanced the cellular uptake by 3.5-folds over that of the 200nm PS nanoparticles. Our investigation clearly demonstrates that the particle size and the particle surface coating are the two most important factors that determine the Caco-2 cell uptake of the nanoparticles and therefore, the feasibility of the nanoparticles to cross the GI barrier for oral chemotherapy.

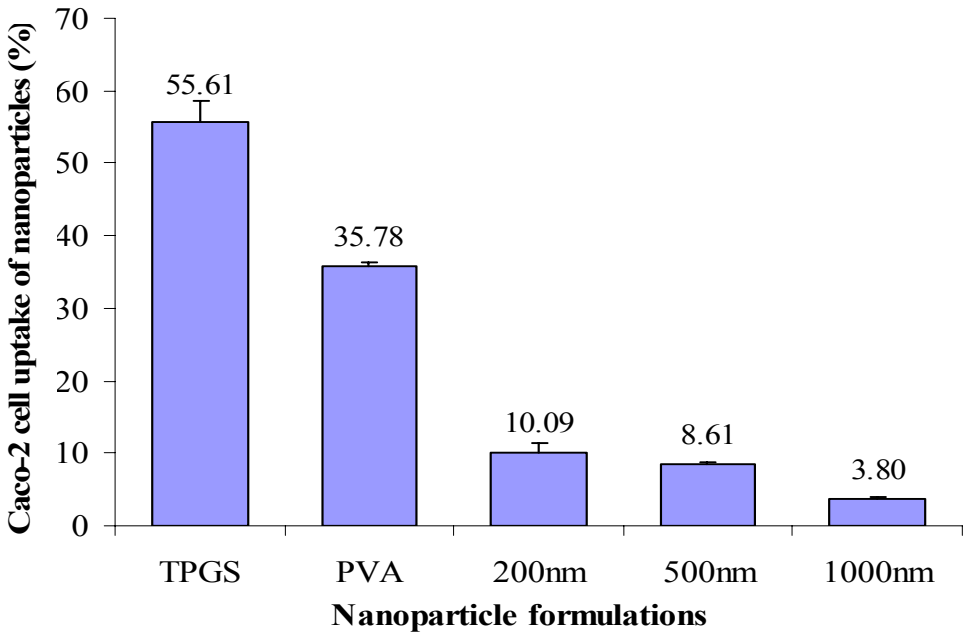
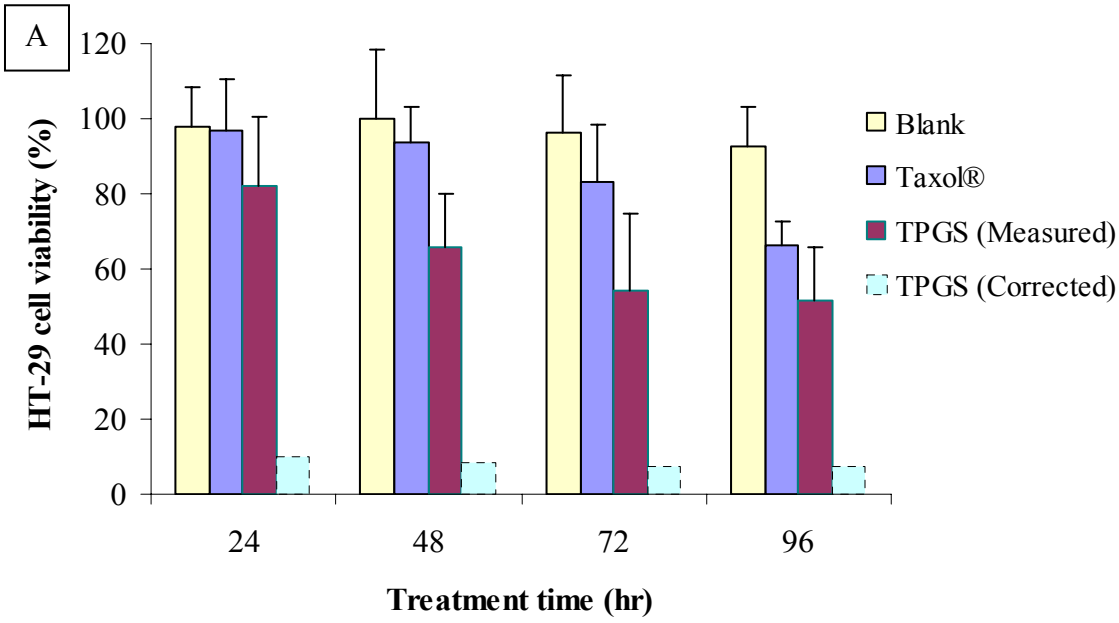


Figure 5. 4. Cellular uptake of standard fluorescent polystyrene nanoparticles with diameter of 200nm, 500nm, 1000nm and PLGA nanoparticles coated with PVA or vitamin E TPGS, which is measured after 2 hours incubation with Caco-2 cells at 37°C. Data represent mean ± SD, n=5.

5.3.5. Cytotoxicity of Nanoparticle Formulation of Paclitaxel

In this study, a human colon cancer cell line (HT-29) was used to determine the anti-tumoral activity of paclitaxel formulated either in the PVA-emulsified or the TPGS-

emulsified PLGA nanoparticles in comparison with its Cremophor EL formulation (Taxol®). Cytotoxic activity of paclitaxel was evaluated by assessing HT-29 cell viability by the MTT assay. The results obtained clearly demonstrate that surface coating of the nanoparticles, the incubation time and the drug concentration play a major role in the *in vitro* cytotoxicity of paclitaxel.



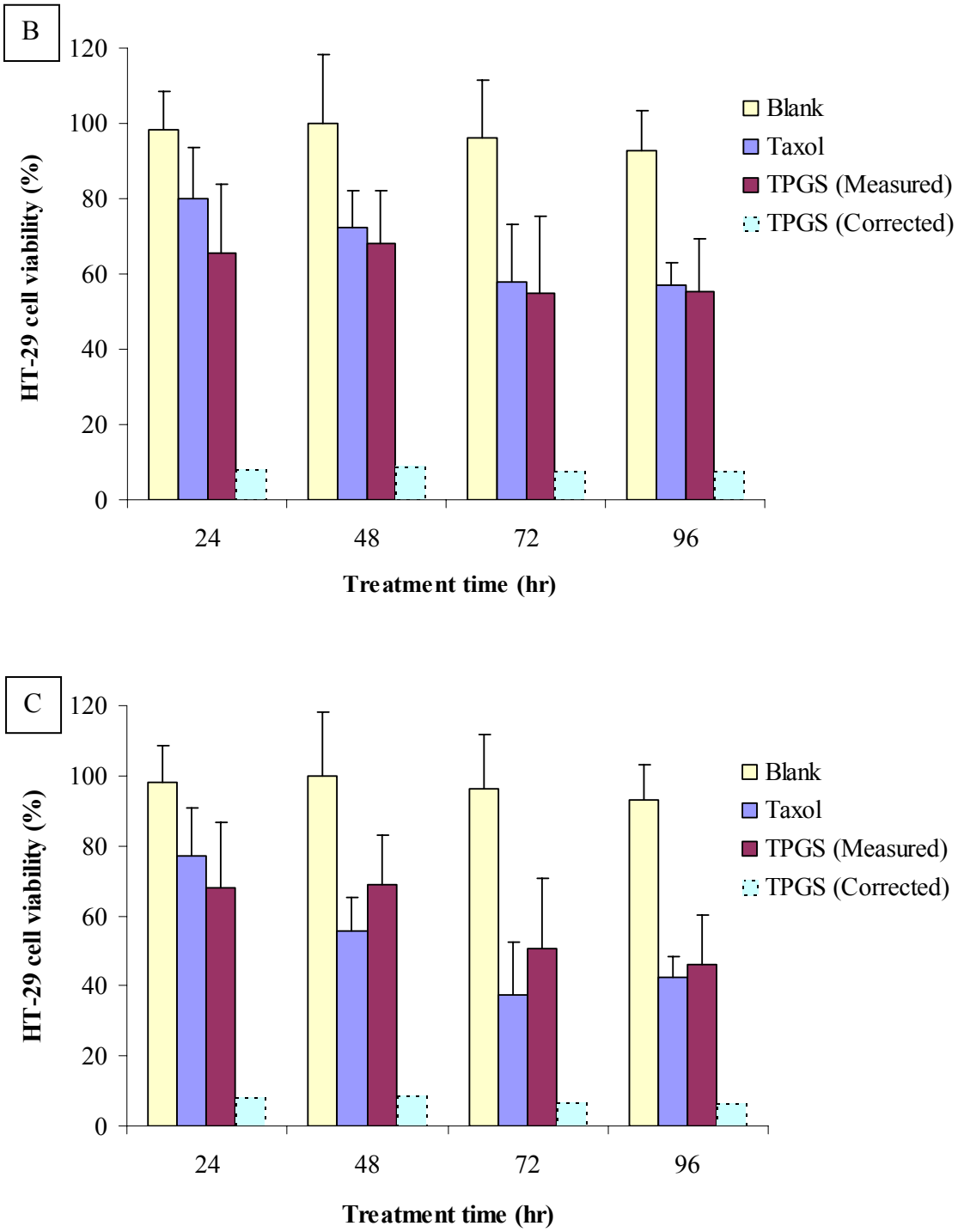


Figure 5. 5. Viability of HT-29 cells indicating effect of the treatment time when incubated with (A) 0.25µg/ml; (B) 2.5µg/ml; and (C) 25µg/ml of paclitaxel in different formulations: Taxol® and vitamin E TPGS-coated PLGA nanoparticles, for 24, 48, 72, and 96 hrs at 37°C. Yellow bars (Blank) represent the viability of control cells and dotted cyan bars (TPGS (Corrected)) for the viability of cells after taking into account of the paclitaxel release from TPGS nanoparticles. Cell viability was determined by the MTT assay and expressed as a percentage of the control wells (cells without treatment). Results shown in this figure represent the mean ± standard deviation obtained for two independent experiments performed with n = 5.

Figure 5.5 shows the viability of HT-29 cells incubated with the same amount of the drug formulated in vitamin E TPGS PLGA nanoparticles or Cremophor EL (Taxol®, 0.25, 2.5 and 25μg/ml of paclitaxel) for 24, 48, 72, and 96 hrs respectively at 37°C, from which a significant reduction in HT-29 cell viability can be observed when the cells were exposed to the TPGS-emulsified nanoparticles in comparison with the Taxol® formulation, especially for the lower paclitaxel concentration. For 0.25μg/ml, the measured value of HT-29 cell viability after 24 hour incubation with the same amount of drug in the Taxol® and in the TPGS-emulsified nanoparticle formulation is 96.81 and 82.01, respectively. Accordingly, the cell mortality is thus 3.19 and 17.99, respectively. Considered that the cumulative release of paclitaxel from the TPGS-emulsified PLGA nanoparticles increased from 0% to 24.42% in these 24 hours, the cell mortality for the TPGS-emulsified nanoparticle formulation should be corrected to be 17.99/(0.2442×0.5) = 147.34 (assuming perfectly linear increase of the drug release). Theoretically, the TPGS-emulsified nanoparticle formulation should thus be 46.18 times (= 147.34/3.19) more effective than the Taxol® formulation. Similar interpretation of the measured data shows that the drug formulated in the TPGS-emulsified PLGA nanoparticle formulation could be 41.64, 19.65, and 10.47 times more effective than that formulated in Cremophor EL (Taxol®) after 48, 72, and 96 hours treatment, respectively. The enhancement of cytotoxic activity of paclitaxel formulated in the nanoparticles can be justified by that the tumor cells can internalize the drug-loaded nanoparticles, allowing the drug to be released inside the cells and therefore, contributing to an increase of the drug concentration near its site of action (Leroux et al., 1996).

For the middle paclitaxel concentration of 2.5μg/ml, the measured cell viability for TPGS-emulsified nanoparticle was comparable to that of Taxol® after 48hrs of

treatment although not 100% of the drug encapsulated was released from the nanoparticles after 48hrs. For the higher paclitaxel concentration of 25μg/ml, Taxol® expressed the lower viability of HT-29 cells. Its higher toxicity was probably attributed by the presence of relatively higher amount of toxic adjuvant, CrEL. However, if the drug release effect was taken into consideration when interpreting the cell viability or mortality of either the middle or the higher drug concentration, the TPGS-emulsified nanoparticles have much higher efficacy comparing to the clinical formulation, Taxol®.

Figure 5.5 also shows the effects of incubation time duration of HT-29 cell viability. As expected, the cell viability was lower for longer periods of incubation with drug or with the drug-loaded nanoparticles, which is consistent with previous reports on the *in vitro* cytotoxicity of paclitaxel against tumor cell lines and in agreement with the mechanism of action of paclitaxel (Raymond et al., 1997; Liebmann et al., 1993; Lopes et al., 1993).

Figure 5.6 shows the effects of the drug concentration on viability of HT-29 cells, which were treated by the drug formulated either in Taxol® or in the TPGS-emulsified PLGA nanoparticles for 24 hrs at 37°C. The drug concentration was chosen to be 0.25, 2.5 and 25μg/ml because this level corresponds to the effective drug concentration in the plasma in chemotherapy (Raymond et al., 1997). It can be seen from Figure 5.6 that there is no noticeable cytotoxicity for the Taxol® formulation at 0.25μg/ml paclitaxel after 24 hrs culture. However, the HT-29 cell viability decreased by 20% at the same drug concentration of 0.25μg/ml but formulated in the TPGS-emulsified PLGA nanoparticles. Significant decreases in the cell viability could be observed as the drug concentration increased from 0.25 to 2.5μg/ml, but further increase in drug concentration did not cause further decrease in HT-29 cell viability for both of the two

formulations. There seems to be a saturate effect as the drug concentration continues to increase. In any case, however, the nanoparticle formulation showed advantages versus Taxol® formulation in *in vitro* cytotoxicity. For 48, 72 and 96 hour incubation, we found similar effects of the drug concentration on HT-29 cell viability (data not shown).

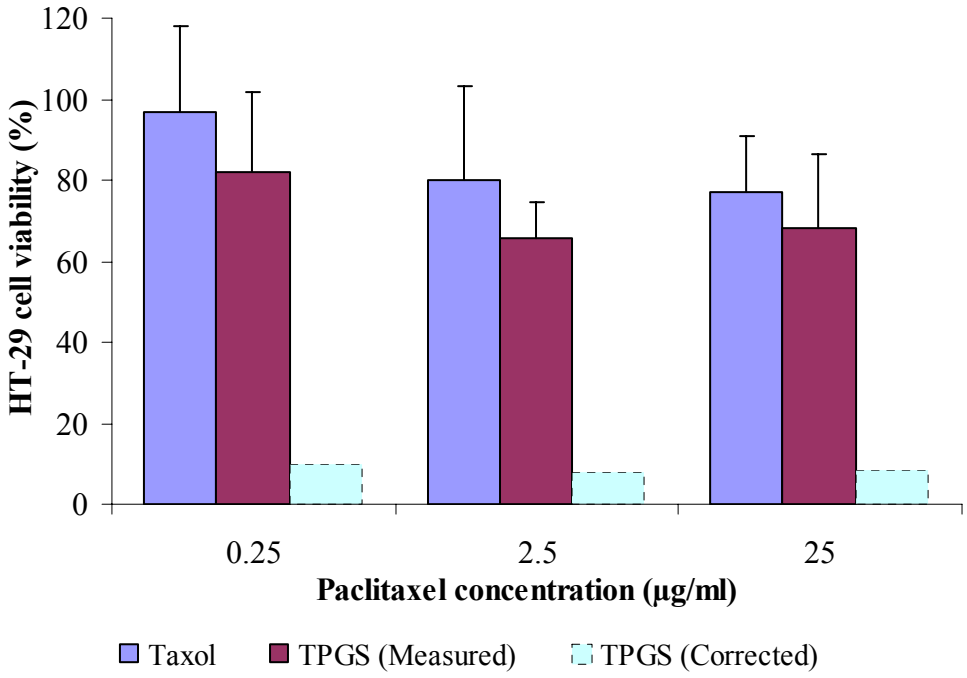


Figure 5. 6. Viability of HT-29 cells indicating effect of paclitaxel concentration formulated in Taxol® (dotted bars) and the vitamin E TPGS-coated PLGA nanoparticles (lined bars), which were treated for 24 hrs at 37°C. Open bars stand for cell viability after corrected with the release of paclitaxel from nanoparticles. Results shown in this figure represent the mean ± standard deviation obtained from two independent experiments performed with n = 5.

5.3.6. Detection of apoptosis sign: intranucleosomal fragmentation

PI staining assay was employed to detect the evidence of paclitaxel-induced apoptotic cell death due to nanoparticle formulation. Nuclear apoptosis can be determined from a series of morphological modifications associated with the execution phase of

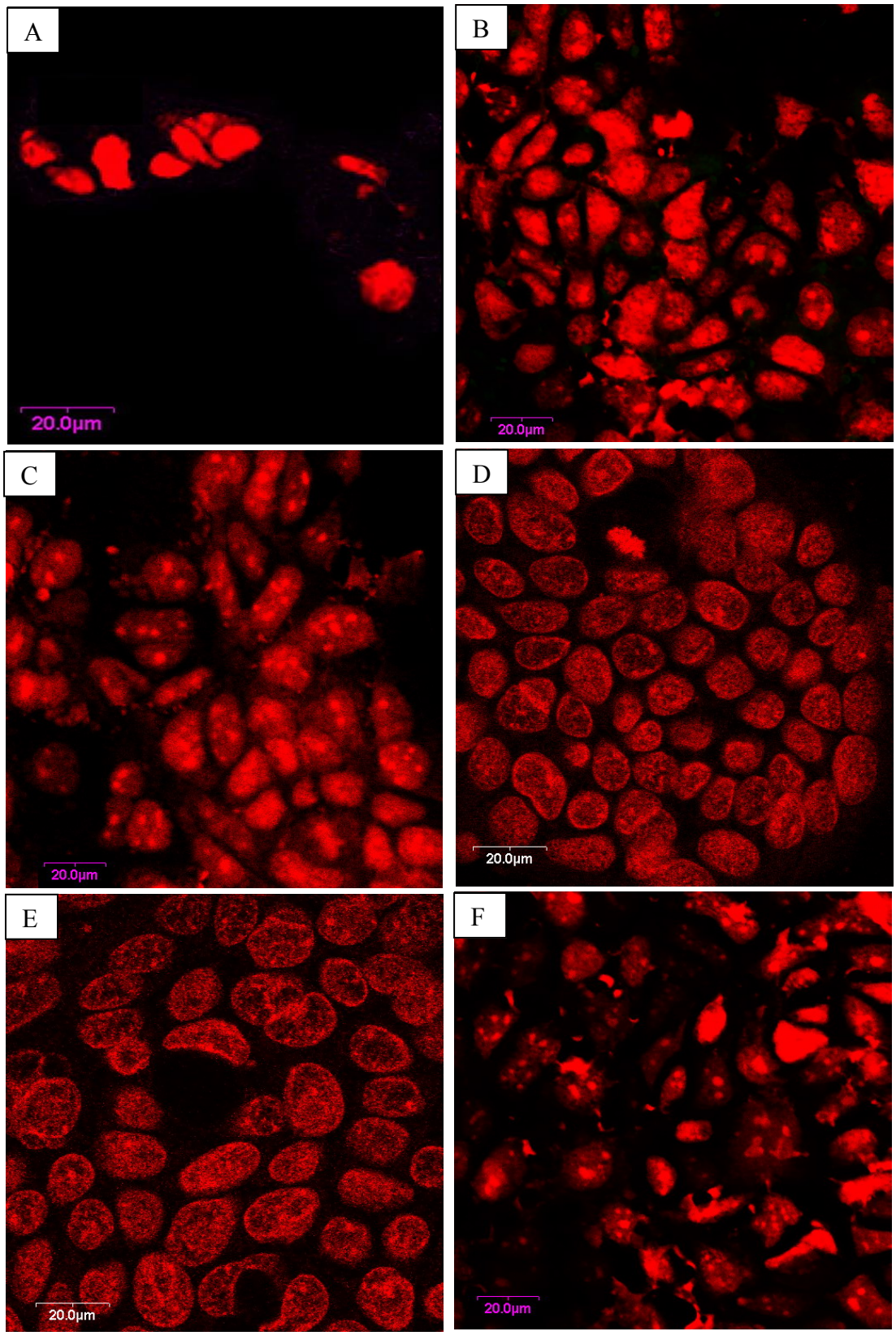


Figure 5. 7. Confocal images of HT-29 cells after incubation with paclitaxel formulations: (A) control; (B) Taxol for 2hr; (C) TPGS-coated nanoparticles for 15min; (D) 30min; (E) 1hr; and (F) 2hrs. Nuclei were stained with propidium iodide. (bar=20µm)

apoptosis (Arends and Wyllie, 1991) which begins with an early perinuclear chromatin condensation leading to massive nuclear destruction. Nuclear apoptosis is accompanied by karyohexis – the segmentation of nucleus into dense nuclear parts which further distributed into apoptotic bodies. This process is a feature of apoptosis and can be detected by analyses such as PI nuclear stain assay and semi-quantitative assay of pulsed-field gel electrophoresis (Walker et al., 1999).

From Fig. 5.7a, the cell nuclei of the control HT-29 cells were seen as homogeneous fluorescence with no evidence of segregation after PI staining. The clinical formulation Taxol® did not produce significant morphological changes in the cell nuclei after treatment of 2hrs (Fig. 5.7b). However, the TPGS-coated nanoparticle formulation lead to fragmentation of the cell nuclei, indicating possible DNA condensation, starting from 15min of treatment time and segregation became apparent after 2hrs of treatment (Fig. 5.7c-f). The nuclear integrity was relatively intact in the cells treated with Taxol® and TPGS-nanoparticles (up to 1hr treatment). Then the severely segregated nuclei was evidenced by the segments of dense fluorescence which suggested a major breakdown in the chromatin followed by DNA condensation in the nuclei.

5.3.7. *In vivo* pharmacokinetics

Figure 5.8 shows the curves of the paclitaxel concentration in plasma versus the time after administration for both of the two formulations. From this figure two key indexes can be obtained. One the so-called area under the curve (AUC), i.e. the graphic area enveloped by the two coordinate axes and the curve of the drug concentration in the plasma versus the time, which is supposed to determine the therapeutic effects of the drug of the given dose in the given formulation.

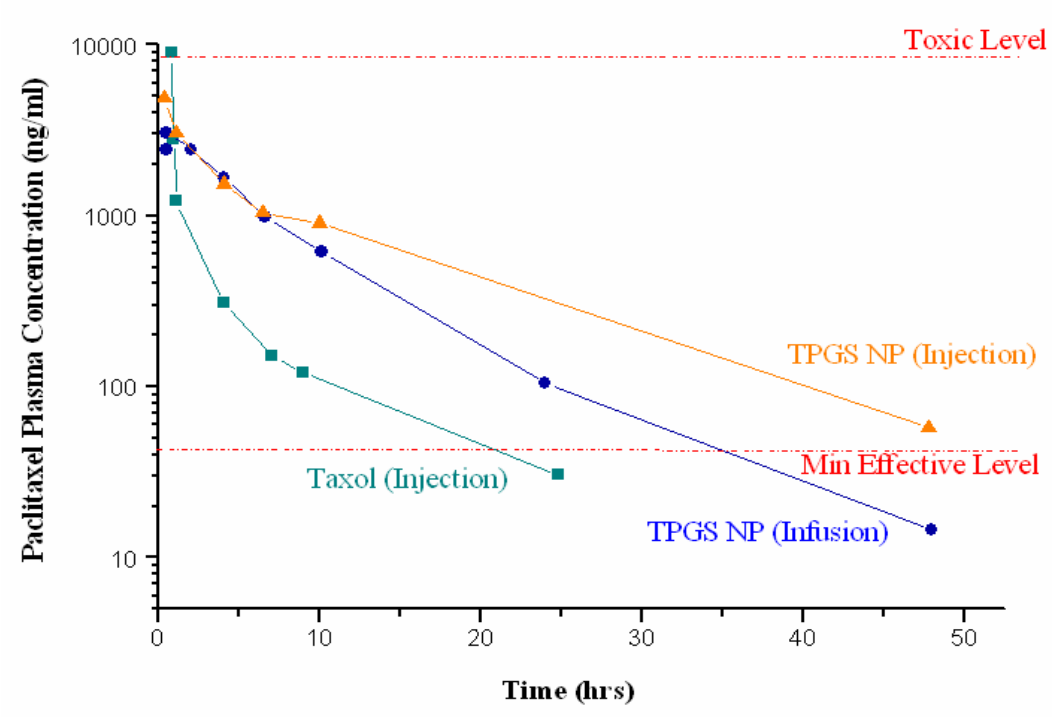


Figure 5. 8. Plasma concentration-time profiles of paclitaxel after i.v. administration to SD rats at 10mg/kg dose formulated in the TPGS-emulsified PLGA nanoparticles and in Taxol®, respectively. The severe side effect level (8,500 ng/ml) and the minimum effective level (43 ng/ml) show the therapeutic window of the drug.

The $AUC_{0\text{-}48hr}$ of paclitaxel in the Taxol® formulation after i.v. injection was 7,018.21ng-hr/ml. The AUC for the nanoparticle formulation was 20,934.56ng-hr/ml after i.v. infusion, which is 3.0 times larger, and 34,291.76ng-hr/ml after i.v. injection, which is 4.9 times larger, respectively. Another important index which can be obtained from Fig. 5.8 is the time at which the drug concentration dropped below the minimum effective value, which for paclitaxel is 50nM or 43ng/ml (molecular weight of paclitaxel is 853.9) (Liebmann et al., 1993). It can be seen from Fig. 5.8 that such a characteristic time is 21 hours for 10 mg/kg dose in the Taxol® formulation, and 35 hours and 50.2 hours for i.v. infusion and i.v. injection, respectively for the same dose in the nanoparticle formulation. This means that the sustainable therapy index for the nanoparticle formulation is 0.59 for i.v. infusion and 0.41 for i.v. injection. Both of the two in vivo indexes show that the drug formulated in the TPGS-emulsified PLGA

nanoparticles can have much higher therapeutic effects that that in the Taxol® formulation. Moreover, Sustainable chemotherapy can be realized by the nanoparticle formulation.

5.4. Conclusion

Vitamin E TPGS-emulsified PLGA nanoparticles were proposed in this study for no-adjuvant formulation as well as for oral delivery of anticancer drugs. Paclitaxel is chosen as a prototype drug due to its high therapeutic effects against a wide spectrum of cancers and its great commercial success as the best seller. The nanoparticle formulation of paclitaxel has great advantages versus the Taxol® formulation. The side effects associated with Cremophor EL contained in the Taxol® formulation can be avoided. In vitro cell viability experiment for HT-29 cells showed much higher cytotoxicity of paclitaxel in the nanoparticle formulation than the Taxol® formulation. The side effects associated with Cremophor EL contained in the Taxol® formulation. The cytotoxicity of the proposed nanoparticle formulation resulted from paclitaxel-induced apoptosis. The nanoparticle formulation can result much higher AUC and thus therapeutic effects of the drug in the nanoparticle formulation than that in the Taxol® formulation. Sustained chemotherapy by nanoparticle formulation can also be realized. Our experiment for cellular uptake of nanoparticles by Caco-2 cells implies that oral chemotherapy by TPGS-emulsified PLGA nanoparticles seems feasible. Our preliminary research shows the power of cancer nanotechnology, which will radically change the way we detect, treat and prevent cancer (NIH and NCI, 2004).

CHAPTER 6

CONCLUSIONS AND FUTURE WORK RECOMMENDATIONS

6.1. Conclusions

The notion of cancer as a serious, life-threatening disease is very ancient. Cancer is a multifactorial disease which can be caused by genetics, lifestyle choices, and exposure to certain environmental hazards. There are about 200 different types of cancer affecting all the different body tissues and each has their own treatment regime. Cancer, in fact, can be cured if diagnosed and treated early; and even if it is at an advanced stage, it can be controlled for months or even years. According to the World Health Organization, more than 10 million people are diagnosed with cancer every year, and it is estimated that this will rise to 15 million new cases every year by 2020. Cancer causes 6 million deaths every year or 12% of deaths worldwide. Nevertheless, no substantial progress can be observed in the past fifty years in fighting against cancer although great effort has been made in cancer research. The death rate by cancer in the United States was 1.939% in 1950 and still 1.940% in 2001.

Chemotherapy is an effective treatment for cancer and other diseases such as cardiovascular restenosis and AIDS, however, it carries a high risk due to the drug toxicity. Problems still exist even for successful chemotherapy as the more effective drugs tend to be more toxic. Thus, patients have to tolerate severe side effects and sacrifice their quality of life. It prompted an intensive research in the past decade in the development of nanoparticles of biodegradable polymers as an effective drug delivery system for improved chemotherapy. In the particulate delivery system, the

drug can be either dispersed in the polymeric matrix, or conjugated/attached to the polymer molecules. After administration, the drug encapsulated in the nanoparticles will be gradually released from the polymer matrix which will eventually degrade into harmless molecules. Nanoparticles of biodegradable polymers can be made of small enough size to allow intracapillary or transcapillary passage and of appropriate coating to allow the particles escape from elimination by the reticuloendothelial system as well as to promote adhesion to, and uptake by, cancer cells.

Paclitaxel is chosen as a prototype drug due to its high therapeutic effects against a wide spectrum of cancers and its great commercial success as the best seller anticancer drug. In fact, paclitaxel is claimed as one of the best anti-cancer drug in the past few decades. However, the limitation of paclitaxel has restricted the dosage and infusion period of the drug for clinical administration purposes. Since best medical effects are yet to be achieved, it becomes necessary to come up with improved formulations of paclitaxel for a more effective clinical administration that eliminates premedication and the toxicity of the adjuvant and also improve efficacy. The desired formulation should be one that does not consume toxic adjuvant, is capable of releasing paclitaxel over an extended period of time with optimum efficacy and stabilizing the compound during long term storage of the formulation but yet economically feasible to be produced on a large scale. Oral paclitaxel is not inherently bioavailable because of the overexpression of P-glycoprotein by intestinal cells and the significant first-pass extraction by cytochrome P450-dependent processes. However, applying nanotechnology and surface modification of the nanoparticles, oral paclitaxel becomes feasible.

In this study, biodegradable polymeric nanoparticles were successfully prepared by oil-in-water emulsification/solvent evaporation process using both chemical and natural emulsifiers. After a process of optimization of fabrication parameters, nanoparticle formulations of a reproducible desirable size with narrow size distribution and high encapsulation efficiency were achieved.

Comparing to chemical emulsifiers, natural surfactants are preferred since they present better performance in preparation and application of polymeric nanoparticles for clinical administration. Among the emulsifiers tested, the natural emulsifiers DPPC and TPGS provide much higher emulsifying efficiency comparing to the chemical emulsifier PVA. This implies that much lower amount of DPPC (about 1/33 of PVA) or TPGS (about 1/67 of PVA) is needed for the same formulation procedures. The fact that TPGS and DPPC have greater tendency to migrate to the surface of nanoparticles has been supported by the XPS analysis of the surface chemistry. XRD analyses revealed that the drug was present in these nanoparticles in an amorphous state. The nanoparticle degradation profile suggested the drug release was mainly due to the drug diffusion followed by polymer matrix degradation. The drug release profile and the degradation pattern are comparable for these TPGS and DPPC-incorporated nanoparticle formulations. Therefore, nanoparticle formulations incorporating TPGS and DPPC were selected as the most suitable formulations for further evaluations.

Pharmaceutical scientists are increasingly turning to the use of cell culture models as an essential tool in high throughput screening assays for absorption and tissue distribution of potential new drug as well as mechanistically to identify specific routes of drug absorption. In this study, Caco-2 cells were used as an *in vitro* model to

evaluate the cellular uptake of nanoparticles coated with vitamin E TPGS or DPPC which aim to promote oral delivery of anticancer drugs. The emphasis was given to the possible effects of particle size and particle surface coating on the cellular uptake of the nanoparticles. Our results illustrated that PLGA nanoparticles have significantly higher level of the cellular uptake compared with polystyrene nanoparticles and the vitamin E TPGS coated PLGA nanoparticles have advantages in favor of the cellular uptake over the PVA coated or DPPC coated PLGA nanoparticles. Thus, TPGS coated PLGA nanoparticle formulation was selected for further detailed studies. An observed plateau effect of the cellular uptake efficiency against the nanoparticle concentration or the cell incubation time suggested that the cellular uptake of polymeric nanoparticle is saturable. In the images of confocal laser scanning microscopy (CLSM), cryo-SEM and transmission electron microscopy (TEM), the internalized nanoparticles were mostly seen throughout the cytoplasm surrounding the nucleus and minority of them in the nucleus, especially in the case of vitamin E TPGS coated PLGA nanoparticles. Our results demonstrated that nanoparticles of biodegradable polymers of small enough size and appropriate surface coating may have great potential to be applied for oral delivery of anticancer drugs as well as other therapeutic agents.

The nanoparticle formulation of paclitaxel has great advantages versus the Taxol® formulation as the side effects associated with Cremophor EL contained in the Taxol® formulation can be avoided. In vitro cell viability experiment for HT-29 adenocarcinoma cells showed much higher cytotoxicity of paclitaxel in the nanoparticle formulation than the Taxol® formulation. The cytotoxicity of the proposed nanoparticle formulation resulted from paclitaxel-induced apoptosis. The nanoparticle formulation can exert much higher AUC and thus therapeutic effects of

the drug in the nanoparticle formulation than that in Taxol® formulation. Sustained chemotherapy by nanoparticle formulation can also be realized. The experimental result of cellular uptake of nanoparticles by Caco-2 cells implies that oral chemotherapy by TPGS-emulsified PLGA nanoparticles seems feasible. Our preliminary research shows the power of cancer nanotechnology, which will radically change the way we detect, treat and prevent cancer.

6.2. Recommendations for future studies

This study is mainly on the formulation optimizations and investigations on their in vitro (thoroughly) and in vivo (a few) efficacy of biodegradable polymeric nanoparticles for clinical administration of paclitaxel. From the results obtained so far, the proposed TPGS coated PLGA nanoparticles strongly demonstrated the feasibility as an efficient drug delivery system. However, it would be more convincing for the clinical application of this proposed formulation if more *in vivo* experiments could be done to support the existing evidences of feasibility.

6.2.1. In vivo pharmacokinetics studies for oral administration of paclitaxel loaded TPGS coated PLGA nanoparticles

To investigate the drug plasma concentration after oral administration of the proposed formulation of paclitaxel, SD rats are orally administered by gavage at a determined dosage of paclitaxel. A group of animals is to be kept as control group. The blood samples are to be taken from the tail vein at predetermined intervals and processed for the LC/MS analysis. The oral bioavailability of paclitaxel can be obtained as the ratio

of the AUC of paclitaxel via oral administration to that of paclitaxel via intravenous injection. All animals were observed for mortality, general condition and potential clinical signs.

6.2.2. Biodistribution of drug studies

The biodistribution study can identify how the drug administered is distributed in the body after administration. Thus, the efficacy of drug delivery system can be further evaluated. In brief, a group of SD rats are administered with paclitaxel formulation and sacrificed at the predetermined intervals. Animals are dissected and organs are removed and processed to determine the drug concentration in individual organs using LC/MS.

6.2.3. *In vivo* evaluation of antitumor efficacy

To decide the efficacy of anticancer drug formulation, antitumor effect of the drug is carried out in mice with weak or no immune response to tumor engraftment. Generally, severely combined immuno-deficient (SCID) mice or athymic nude mice (nu/nu) are used. To develop the tumor model, HT-29 cells are subcutaneously injected into the mice. Tumor nodules are allowed to grow to a volume > 150mm^3 prior to the experiment. Tumor-bearing mice are grouped for each type of treatment and drug formulations are administered. Using a digital caliper, the length and width of tumor are measured in millimeters and tumor size can be calculated using [length×(width×width)/2] and expressed in milligrams. The body weights and tumor volume of each group are monitored thrice weekly.

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Win KY, Feng SS. 2005. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials 26, 2713-2722.

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APPENDIX A

LIST OF PUBLICATIONS

1. Internationally refereed journals

- Wang J, Ng CW, Win KY, Shoemakers P, Lee TKY, Feng SS, Wang CH. **2003**. Release of Paclitaxel from Polylactide-co-glycolide (PLGA) Microparticles and Discs under Irradiation, *J. Microencapsulation* 20(3), 317-327. (NUS Tier 2, JIF = 1.370)
- Feng SS, Mu L, Win KY, Huang G. **2004**. Nanoparticles of Biodegradable Polymers for Clinical Administration of Paclitaxel, *Current Medicinal Chemistry* (invited paper) 11(4), 413-424. (NUS Tier 1, JIF = 4.904)
- Win KY, Feng SS. **2005**. Effects of Particle Size and Surface Coating on Cellular Uptake of Polymeric Nanoparticles for Oral Delivery of Anticancer Drugs, *Biomaterials* 26(15), 2713-2722. (NUS Tier 1, JIF = 4.698)
- Win KY, Feng SS. **2006**. In vitro and in vivo studies on vitamin E TPGS-emulsified poly(D,L-lactic-co-glycolic acid) nanoparticles for paclitaxel formulation, *Biomaterials* 27(10), 2285-2291. (NUS Tier 1, JIF = 4.698)

2. Book chapter

- Feng SS, Lee PZ, Win KY. **2006.** Nanoparticles of Biodegrdable Polymers for Cancer Chemotherapy. In *Nanoparticles for Pharmaceutical Applications* (Eds. A. J. Domb, Y. Tabata, M. N. V. Ravi Kumar), American Scientific Publisher, Valencia, California 91381-0751, USA.

3. International conferences

- Wang JJ, Ng CW, Win KY, Shoemakers P, Lee T, Feng SS, Wang CH. Controlled Release of Paclitaxel from Spray Dried Polylactideco-glycolide (PLGA) Microparticles. *6th World Congress of Chemical Engineering*, September 23-27, **2001**, Melbourne, Australia.
- Foo SH, Win KY, Feng SS, Wang CH. Characterization of Paclitaxel-loaded Biodegradable Particulate Systems. *AIChE 2002 Annual Meeting*, November 3-8, **2002**, Indiana, USA.

- Win KY, Mu L, Wang CH, Feng SS. Nanoparticles of Biodegradable Polymers for cancer Chemotherapy. *2003 Summer Bioengineering Conference*, June 25-29, **2003**, Florida, USA.
- Win KY, Wang CH, Feng SS. Coatings of Paclitaxel-loaded Particles Enhance the Release of Drug and Improve Cell Uptake. *30th Annual Meeting & Exposition of the Controlled Release Society*, July 19-23, **2003**, Glasgow, Scotland.
- Feng SS, Mu L, Win KY. Nanoparticles of Biodegradable Polymers for Clinical Administration of Anticancer Drugs: Chemotherapeutic Engineering in Singapore. *World Congress on Medical Physics and Biomedical Engineering (WC2003)*, August 24-29, **2003**, Sydney, Australia.
- Feng SS, Win KY. Vitamin E TPGS Emulsified Nanoparticles of Biodegradable Polymers for Oral Delivery of Paclitaxel. *31st Annual Meeting & Exposition of the Controlled Release Society*, June 12-16, **2004**, Hawaii, USA.
- Win KY, Feng SS. Nanoparticle Technology for Oral Chemotherapy. *1st Nano-Engineering and Nano-Science Congress,* July 7-9, **2004**, National University of Singapore, Singapore.
- Feng SS, Win KY. Nanoparticles of Biodegradable Polymers for New-Concept Chemotherapy. *96th Annual Meeting of American Cancer Research*, Apr 16-20, **2005**, California, USA.

APPENDIX B

LIST OF ACHIEVEMENTS

Ranked by **ScieceDirect.com** as **TOP 25 Hottest Paper**

- Win KY, Feng SS. **2005**. Effects of Particle Size and Surface Coating on Cellular Uptake of Polymeric Nanoparticles for Oral Delivery of Anticancer Drugs, *Biomaterials* 26(15), 2713-2722. (NUS Tier 1, JIF = 4.698)
  - At **6th** position in the journal of **Biomaterials**, Q4 2005
  - At **17th** position in the journal of **Biomaterials**, Q2 2005
  - At **5th** position in the journal of **Biomaterials**, Q1 2005

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