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Division of Molecular Therapeutics and Formulation School of Pharmacy,
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**Improving Nanoparticle Drug loading: Matching Polymer
to Drug Properties and making of Polymer coated Drug
Nanoparticles**

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*'To my late dad Alhaji A.A Sodeinde (owo white); my first teacher, who taught me to let
my pen breathe'*

As a child I always wanted to learn about medicines, I was intrigued by the ‘magical substances’ we took when we fell ill, that made us well. After graduating as a pharmacist, in 2008, I practised as a clinical Pharmacist in Nigeria, I found out first-hand the limitations of conventional drug delivery approaches to some difficult-to-treat diseases. This even came closer to home when my half-brother was diagnosed with cancer and we found out that he stood no chance. He got worse quickly despite chemotherapy and surgery. Sometimes, we felt the side effects were worse than the actual disease. I was disappointed in my so-called magical substances.

When I joined the pharmaceuticals department of the faculty of pharmaceutical sciences of the University of Ilorin, in 2012, I immediately wanted to seek further knowledge on the science behind making medicines. This was further informed by the need to look beyond systemic drug administration for diseases like cancer where there was need for selectivity and controlled release of drugs. In addition to this, there was a realisation that the successful use of drugs depended a lot on actually getting enough of them to the right place.

My life experiences directed me towards this path. A path of learning advanced drug delivery techniques and learning more about delivering drugs in the smartest way. Furthermore, I wanted to learn how to tailor the new scientific knowledge to diseases that were more common in Africa and also harness the extensive natural resources available.

With my part-funding in hand, I packed up my bags and started this journey with my two little children. It has been absolutely rewarding albeit extremely challenging and difficult. We went through it all and are quite proud of where we are now. Although it has become drawn out and emotionally draining, the positives still far outweigh the negative as I look forward to a successful end to one phase and introduction to the next!

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Abstract

Poor Central Nervous System (CNS) penetration by cancer drugs limits their application in brain tumours, such as medulloblastoma. An alternative approach, delivering anticancer drugs directly to residual tumour using nanoparticulate delivery systems (NPDS) could reduce some of these problems. The main challenges hindering the clinical translation of the use of NPDS for local delivery of drugs to the residual tumour are inadequate drug loading and erratic release.

This study focusses on understanding the conditions required for the development of a NPDS with sufficient drug loading for post-surgical delivery to the residual tumour. Known effective drugs; Etoposide, Etoposide phosphate and Teniposide were screened against modified poly(glycerol) adipate based polymers. This was done by monitoring drug release from 40% drug-polymer loaded films and using Fourier Transform Infra-Red spectroscopy (FTIR) and contact angle measurements to explain the release results. Polymers of different structure were then matched with the specific drugs based on the interactions observed to prepare drug loaded nanoparticles.

Two types of drug loaded nanoparticles were prepared from these combinations. Drug loaded matrix polymer nanoparticles (NP) were prepared by an interfacial deposition method, while a novel process of applying a polymer coating to drug nanoparticles, termed polymer coated drug nanoparticles (PCDNP), was also developed. The nanoparticles were characterised applying physicochemical characterisation methods, and their cytotoxicity assessed.

The key benefits of this work are the development of a systematic method to match polymers to drugs through assessing drug and polymer interactions, resulting in the formulation of nanoparticles with higher

drug loading, and secondly the development of a novel technique of making nanoparticles, polymer coated drug nanoparticles (PCDNP).

Abbreviations

ATR	Attenuated Total reflection
BBB	Blood-brain barrier
BP	British pharmacopoeia
CA	Contact angle
CAB	Carbamic acid benzyl ester
CBTRC	Children's brain tumour research centre
CED	Convention enhanced delivery
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Chemotherapy
DCM	Dichloromethane
DDS	Drug delivery systems
DLPNP	Drug loaded polymer nanoparticle
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
DS	Delivery system
EDL	Electrical double layer
EDTA	Ethylenediaminetetraacetic acid
ELS	Electrophoretic light scattering
EPR	Enhanced permeability and retention
FBS	Fetal Bovine Serum
FDA	Food and drug administration
FTIR	Fourier transform infrared spectroscopy
HGG	High grade glioma

HP- β -CD	Hydroxypropyl- β -cyclodextrin HP- β -CD
HPC	Hydroxypropyl cellulose
HPLC	High performance liquid chromatography
HPMC	Hydroxyl propyl methylcellulose
HS-15	Solutol
IC ₅₀	Half maximal inhibitory concentrations
IDP	Interfacial deposition
IV	Intravenous
kDa	Kilodalton
LNC	Lipid nanocapsule
LOD	Limit of detection
LOQ	Limit of quantitation
MB	Medulloblastoma
MCT	Medium chain triglycerides
MMA	Methyl methacrylate
MPEG-PCLA	Monomethoxy poly(ethylene glycol)-poly (ϵ - caprolactone-co-D,L- lactide)
MPS	Mononuclear phagocyte system
MRI	magnetic resonance imaging
NIBS	Non-Invasive Back-scatter
NMR	Nuclear magnetic resonance
NP	Nanoparticles
NPDS	Nanoparticulate delivery system
OH	Hydroxyl
P188	Poloxamer 188
PBCN	Poly(butyl cyanoacrylate)
PBS	Phosphate buffered saline
PEG-PSA	poly(ethylene glycol)-co-poly(sebacic acid)
PHBHHx	poly-3-hydroxybutyrate-co-3-hydroxyhexanoate

PCDNP	Polymer coated drug nanoparticle
PCNP	Polymer coated nanoparticle
PCL	Polycaprolactone
PCL-PEG	Poly(caprolactone)-PEG
PCI-PEGMA	poly(caprolactone)-co-poly(ethylene glycol methacrylate)
PCS	Photon correlation spectroscopy
PDLLA	Poly(D,L-lactide)
PDL-PEG	Poly(decylactone)-PEG
PEG	Polyethylene glycol
PGA	Poly(glycerol adipate)
PGA _{40%} C ₁₈	40%C ₁₈ substituted
PGA _{40%} C ₈	40%C ₈ substituted
PGA _{85%} C ₁₈	85%C ₁₈ substituted
PGA-30%CH	30%Cholesterol substituted
PGA-Phe	50%Phenylalanine substituted
PGA-SA	50%Succinic anhydride substituted
PGA _{50%} TRY	50% Tryptophan substituted
PLA	Poly lactides
PLA-PEGMA	Poly(lactic acid)-co-poly(ethyleneglycolmethacrylate)
PLGA	Poly(lactic-co-glycolic acid) `
PP	Paediatric prevalence
PI	Peak incidence
PVA	Polyvinyl alcohol
PVCL	Poly(N- vinyl caprolactam)
RBITC	Rhodamine B Isothiocyanate
SEM	Scanning electron microscopy
SHH	Sonic hedgehog
SLN	Solid lipid nanoparticle

TEM	Transmission electron microscopy
TENI	Teniposide
THF	Tetrahydrofuran
TPGS	Tocopheryl polyethylene glycol 1000 succinate
UF	Ultrafiltration
UV	Ultraviolet
WHO	World health organisation
WNT	Wingless
ZP	Zeta potential

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Chapter 1

1 Introduction

This thesis describes work carried out to study ways to improve nanoparticle drug loading by matching polymer to drug properties, with a view to making of polymeric matrix nanoparticles with increased drug loading. The thesis also describes a new method of polymer coated drug nanoparticles production. The target tumour type that is selected is medulloblastoma as it is the most common malignant brain tumour of children and has established sensitivity to a range of systemically and locally administered cytotoxic drugs. Successful development of a drug delivery system could hence be anticipated to enhance the efficacy and reduce the toxicity of current therapies. The nanoparticle delivery system envisaged, is being developed to address the particular requirement of childhood malignant brain tumours, like medulloblastoma, which have the propensity of both growing locally and disseminating within and beyond the CNS. The introduction will, therefore, describe the selected target tumour and its relevant characteristics. It will then describe nanoparticulate delivery systems, their application in drug delivery and the challenges preventing successful translation of research into the clinic. The introduction will also identify existing drug delivery systems that are currently used in clinical practice and consider the different approaches and techniques for introducing drugs, or delivery systems, to the brain. This introduction will be concluded with a more detailed description of the anticancer drugs selected for this work, the proposed delivery system, the desired properties in the proposed nanoparticle formulations, and the specific aims of the project.

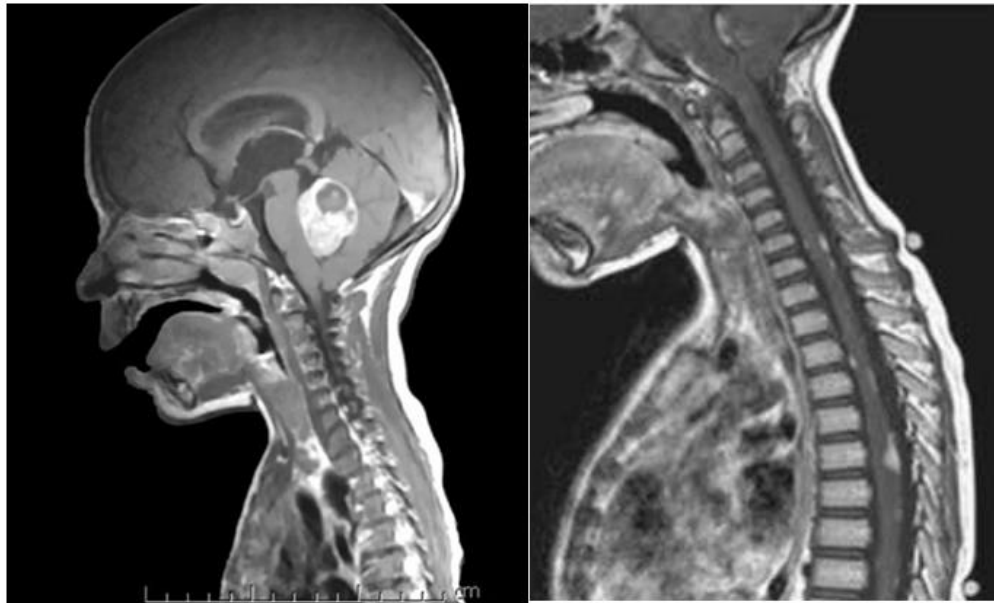
1.1 Medulloblastoma

Medulloblastoma (MB) is a malignant neuroectodermal tumour of the cerebellum, a posterior fossa structure, which occurs in the childhood age range ((1). MB accounts for 16-20% of childhood tumours of the central nervous system (CNS) and because of its malignant nature is the leading cause of tumour-related deaths in children (1,2). Anatomically, it arises either in the midline involving the 4th ventricle or less commonly within the cerebral hemispheres. The overall age incidence curve peaks at age 4-8 years, although the molecular subtypes have different age incidence curves within childhood, supporting the hypotheses that this is a group of tumours arising as a consequence of embryological events linked to brain growth and development. It does occur in adults with an incidence rate ratio of 4.61/1 (children/adults) (3) and is more common in males by a ratio of 1.56:1 (4).

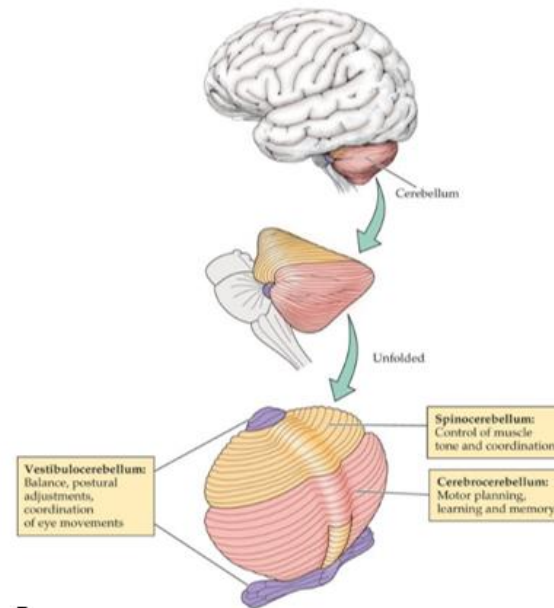
Although MB arises within cerebellar structures, local brain invasion and dissemination via cerebrospinal fluid (CSF) pathways to other parts of the brain and spinal cord do occur. Dissemination beyond the CNS also does occur (5). CNS metastases are found in 30-40% of patients at the point of diagnosis (6). Dissemination to the leptomeninges has been assumed to be via the CSF pathways (7). Although, Garzia *et al* recently hypothesized that that recurrence of MB could be *via* the vascular route (8). The factors predicting worse outcomes at the time of diagnosis include greater than 1.5 cm² of post-operative residual tumour on 2-dimensional imaging, diagnosis under 3 – 5 years of age and the presence of metastases (2,4).

Patients typically present with symptoms linked to raised intracranial pressure due to the presence of tumour filling the fourth ventricle causing local swelling and obstruction of the drainage of CSF from supratentorial ventricles leading to obstructive hydrocephalus. This causes nausea, vomiting and headache and in young children under two years of age,

rapidly accelerating head growth (9). A false localising sign can occur due to raised pressure causing paralysis of the 6th cranial nerve leading to a paralytic squint and double vision. Tumour invasion and compression of cerebellar structures and connecting cerebellar tracts cause ataxia and an associated unsteady gait. In younger children, this local swelling can also cause neck pain and a head tilt which can be confused for the self-limiting condition of torticollis or wry neck.



A



B

Figure 1-1 MRI image of a patient with medulloblastoma B: Image of the cerebellum and the functions, online image taken from (10).

1.2 Classification of Medulloblastoma

According to the world health organisation (WHO) revised classification in 2016, MB is classified both histologically and by genetic characteristics (11).

1.2.1 Histological classification:

- I. Classical: This class is the most common. The tumours are made up of sheet-like densely packed areas of small round cells, with scanty cytoplasm and a dense hyperchromatic nucleus (6).
- II. Desmoplastic: This class is seen in 10-15% of the case. The tumours are made up of scattered round nodules separated by nodules rich in reticulin. This class makes up about 50% of adult cases of MB but 15% of cases is found in children.
- III. Large cell or anaplastic: This accounts for 4% of cases and is the most malignant MB variant. Tumours contain large cells with pleomorphic nuclei, abundant cytoplasm and prominent nucleoli. This variant is associated with poor prognosis as it confers resistance to primary adjuvant therapy with radiotherapy and chemotherapy.
- IV. Melanotic: This class is made up of accumulated melanotic tumour cells.
- V. Medullomyoblastoma: This class is made up of areas of focal myogenic differentiation.

1.2.2 Genetic subtypes

There are four genetic (molecular) groups, known as Wingless (WNT), sonic hedgehog (SHH), group 3 and group 4. There are a number of possible and genetic combinations. A summary of some of the most

common combined medulloblastoma diagnoses (see Table 1-2) was provided by Louis *et al* (12).

MB has been staged, based on the presence or location of metastasis (4). When there is no evidence of metastasis, Mo staging is given to the disease. M₁ is given when there are malignant cells in the cerebrospinal fluid (CSF). M₂ and M₃ are given for when malignant cells spread to the cerebral subarachnoid space and spinal subarachnoid space respectively, while M₄ is for extra-neural spread. Risk classification is used clinically and is critical to the management of MB. It is made based on age, amount of residual tumour and metastasis (13) (Table 1-1).

Classification	Description
High risk	Metastatic, residual tumour greater than 1.5 cm ² and age of diagnosis is below 3 years
Average risk	No metastasis, residual tumour less than 1.5 cm ² and age of diagnosis is 3 years and above
M ₀	No evidence of tumour spread
M ₁	Evidence of metastasis to the cerebrospinal fluid
M ₂	Evidence of metastasis within the brain
M ₃	Tumour spread to the spine
M ₄	Metastases beyond the CNS

Table 1-1 Classification of Medulloblastoma based on metastases (M₁ – M₄)(14) and clinical presentation (15,16).

Genetic profile	Histology	Prognosis
WnT activated	Classic Large cell/anaplastic (very rare)	Low risk tumour Tumour of uncertain clinicopathological significance
SHH activated	Classic Large cell/anaplastic Desmoplastic/nodular(very rare)	Uncommon high risk tumour High risk tumour; prevalent in children aged 7 – 17 years Tumour of uncertain clinicopathological significance
Non WnT/non SHH Group 3	Classic Large cell/anaplastic	Standard risk tumour High risk tumour
Non WnT/non SHH Group 4	Classic Large cell/anaplastic	Standard risk tumour; Tumour of uncertain clinicopathological significance

Table 1-2 Summary of some of the most common combined medulloblastoma diagnosis and prognosis, taken from (12)

1.3 Treatment

The treatment of MB is multimodal involving surgery, radiation and chemotherapy (17). The first line of treatment is surgery is aimed at:

1. Controlling raised intracranial pressure by inserting a ventriculo-peritoneal shunt or bypassing CSF flow obstruction with a 3rd ventriculostomy.
2. Obtaining tumour tissue for histological and biological evaluation.
3. Removing as much tumour as possible while minimising damage to neurological function (5).

Complete resection sometimes poses a risk of morbidity due to the proximity of the tumour to the fourth ventricle and or the brain stem (4). The target is to have residual tumour mass of less than 1.5 cm² on 2-dimensional imaging (6). One significant brain injury risk, peculiar cerebellar tumours and MB in particular, is the risk of the patient developing mutism within a few days of surgery, this is called cerebellar mutism and is reported to occur in up to 30% of cases (6). Where other focal neurological damage is apparent before or after surgery, persistent ataxia, speech and language apraxia and reduced speed processing speed can complicate subsequent cognitive functioning, quality of life and impair complete rehabilitation.

Craniospinal radiation is recommended to be administered immediately after surgery (3-6 weeks) depending on the age of the patient. The irradiation of the craniospinal axis is done to eliminate residual tumours undetectable in the CSF or with magnetic resonance imaging (MRI) and therefore to reduce the risk of reoccurrence (18). The introduction of this strategy improved the 5-year survival rate of the patients to about 40% (19). Prognosis was further improved when a post-operative radiotherapy prescription of 36-39 Gy was introduced to the whole CNS followed by a

posterior fossa boost of 50-54 Gy (20). This, however, led to significant toxicity which includes neurocognitive decline, endocrinopathies, impaired spinal growth, late effects on neuro-vasculature with enhanced risk of stroke in adult life (4,5). These late toxicities have a significant impact on the quality of life of survivors (20). Craniospinal radiotherapy was associated with greatly exaggerated toxicities and so its use is avoided in children below 3 years (4).

In the light of these toxicities and their long term effects, a strategy seeking to improve treatment was to optimise adjuvant treatment by reduction of the radiation dose and adding chemotherapy (20) with the intent to sustain survival whilst reducing late toxicities. This has been associated with a sustained improvement in overall survival rates. Most recently, the introduction of more focussed radiation techniques such as tomotherapy and proton therapy has reduced the total volume of normal tissues exposed to higher radiation dose which is hoped to reduce late consequences for subsequent tissue growth, function and second cancer risk (21-23).

Chemotherapy (CT) has been used in the treatment of MB to not only reduce the amount of radiation required but to also contribute to the progression-free survival of MB patients (13). Some of the most commonly used drugs are high dose methotrexate, etoposide, cisplatin, cyclophosphamide, vincristine, carboplatin and nitrosourea (20). The addition of chemotherapy has been shown to reduce the dose of craniospinal radiation, thereby reducing the dose-dependent high toxicity of radiation (6). A randomised trial done on children with MB showed event-free survival of 81% in standard-risk patients who received 23.4 Gy RT and chemotherapy (2 possible regimens comprising lomustine, cisplatin and vincristine or lomustine, vincristine and cyclophosphamide at varying dosing intervals) (24). A similar result was obtained with concurrent administration of chemotherapy with reduced RT dose (25).

The authors reported 86% disease-free survival in average-risk patients. These results show that chemotherapy can improve survival while reducing the amount of radiation dosing (26).

At this stage a recent consensus statement identified that by combining staging characteristics and molecular subtyping, risk groups could be defined based on survival rates: low risk (>90% survival), average (standard) risk (75–90% survival), high risk (50–75% survival) and very high risk (<50% survival) disease. The WNT subgroup and non-metastatic Group 4 tumors with whole chromosome 11 loss or whole chromosome 17 gain were recognized as low-risk tumours. High-risk strata were defined as patients with metastatic SHH or Group 4 tumours, or MYCN amplified SHH medulloblastomas. Very high-risk patients are Group 3 with metastases or SHH with TP53 mutation. This risk stratification opens the way to adapt treatment to the subgroups seeking to reduce toxicity in low-risk cases and intensify treatment in very high-risk cases. Such a strategy requires the application of therapies to either augment or replace craniospinal radiotherapy.

1.3.1 Obstacles to chemotherapy

Drug therapy, whether with cytotoxic or biologically active agents, is the focus of much research. Experience suggests that cytotoxic chemotherapy plays a significant role in the successful treatment of MB. However, in considering strategies to enhance its efficacy, by site-specific drug delivery, several obstacles impair efficient drug therapy.

1.3.1.1 Side effects of chemotherapeutic agents

The advantages of the addition of chemotherapy to treatment are not maximised due to many side effects (27). The side effects of chemotherapy

stem from the inability of the drugs to differentiate between healthy and diseased cells and also the high dose given to ensure adequate effect (28).

Anticancer chemotherapeutic agents mainly act on rapidly dividing cancer cells and this causes toxicity because they affect other healthy cells that are also rapidly dividing (20). Examples of rapidly dividing cells include haematopoietic cells, the skin and its appendages and cells of the gastrointestinal tract. This is responsible for some of the most common side effects of chemotherapy like anorexia, nausea, vomiting and myelosuppression (29). Side effects are made worse by the high dose of drugs administered systemically to compensate for poor drug distribution to unintended sites.

1.3.1.2 The characteristics of the tumours and their microenvironment

A second obstacle to successful chemotherapy to the brain is the heterogeneity of the tumour microenvironment. The tumour microenvironment is made up of the tumour, the surrounding vascular network, extracellular matrix, and normal non-malignant cells (30,31). Tumour development and progression are driven by the mutation of the cancer stem cells. In normal tissue the stroma acts as a barrier to tumour generation however, the tumour stroma is altered by the presence of the different cell types which play a major role in angiogenesis and consequently tumour growth and malignancy (32). These cell types include the endothelial cells, fibroblasts, immune cells and are influenced by extracellular components like cytokines, growth factors and hormones. Heterogeneity because of these components and the proteins they secrete vary with tumour type and disease stage (33). All of which makes tumour targeting and specificity difficult.

This complex tumour microenvironment also poses a physical barrier to successful chemotherapy due to the architecture of the tumour tissue and the organisation of the tumour microenvironment. The response to chemotherapy is different in tumour associated endothelial cells due to changes in proliferation, migration and responses to growth factors. The different cell populations may contribute individually to the traits expressed by malignancies and their ability to metastasize (34).

A combination of the changing vasculature of the tumour microenvironment leads to a pressure differential between the core and outside edge of the tumour, hypoxia and acidity, and may lead to unpredictable drug distribution to the tumour core and differences in therapeutic efficacy with tumour type and patient. The abnormal vasculature present in tumours affects perfusion leading to hypoxia which may negatively impact drug delivery (35). Hypoxia also affects the interstitial pH which may affect the ionization of the chemotherapeutic agents which would affect their uptake (36). The higher interstitial pressure in tumours than normal tissues could lead to the increased outward flow of components to the surroundings (35). The distance of the tumour core to the vascular bed may also limit drug distribution to the core. It has been identified that the alterations in the fibroblasts secreted factors and signalling, immune response and the tumour vasculature may play a role in the mechanism of resistance to chemotherapy (37).

For medulloblastoma, heterogeneity of the different subgroups due to their distinct histology and demographics may affect clinical outcomes. The presence of these subgroups raises the need to treat MB as four different diseases (38). The different molecular subgroups of MB may have different response rates to drugs (1). In addition to this, relapse is mainly as a result of metastasis which affects risk classification and prognosis. Zapotocky *et al* concluded from their recent study that pattern of

metastasis may be associated with the molecular biology of the tumours and this may affect treatment outcome (39).

Evidence also shows that medulloblastoma recurrence may involve clonal selection of a minor clone which may have existed at the time of diagnosis (40). This raises the possibility that recurrent tumours are different from their tumour of origin (38). Hence, treatment options have to be chosen with careful consideration for the heterogeneity based on subtypes. The implication of this is that heterogeneity makes tumour response to treatment difficult to predict (41) and the metastatic tumour may require a separate study and treatment development.

1.3.1.3 Blood Brain Barrier (BBB)

The treatment of brain tumours is further made difficult by the presence of the blood brain barrier (BBB) (42). The BBB is a complex physiological, anatomical and biochemical structure (1). This barrier serves to selectively prevent penetration by substances from the blood into the brain. Small, neutral, and lipid-soluble molecules normally readily penetrate the BBB, however, many chemotherapeutic agents do not fall into this category. This protective role of the BBB leads to a severe limitation of the movement of drugs into the brain.

The BBB is made up of densely packed endothelial cells held together side by side by tight and adherens junctions with no fenestrations (43). The endothelial cells are lined by pericytes, astrocytes and basement membrane. The pericytes wrap around the capillaries to provide structural support, while the astrocytes and basal lamina surround the endothelial cells and pericytes (Figure 1-2). They function to maintain chemical stability and allow interactions with neurons. The specific role of the tight junctions is to prevent diffusion of **blood-borne substances** between the cells. The BBB is also involved in active metabolism and

carrier-mediated transports. The presence of efflux proteins, like P glycoprotein and ABCG2, in the BBB impedes drug action by pumping out chemotherapeutic agents (44).

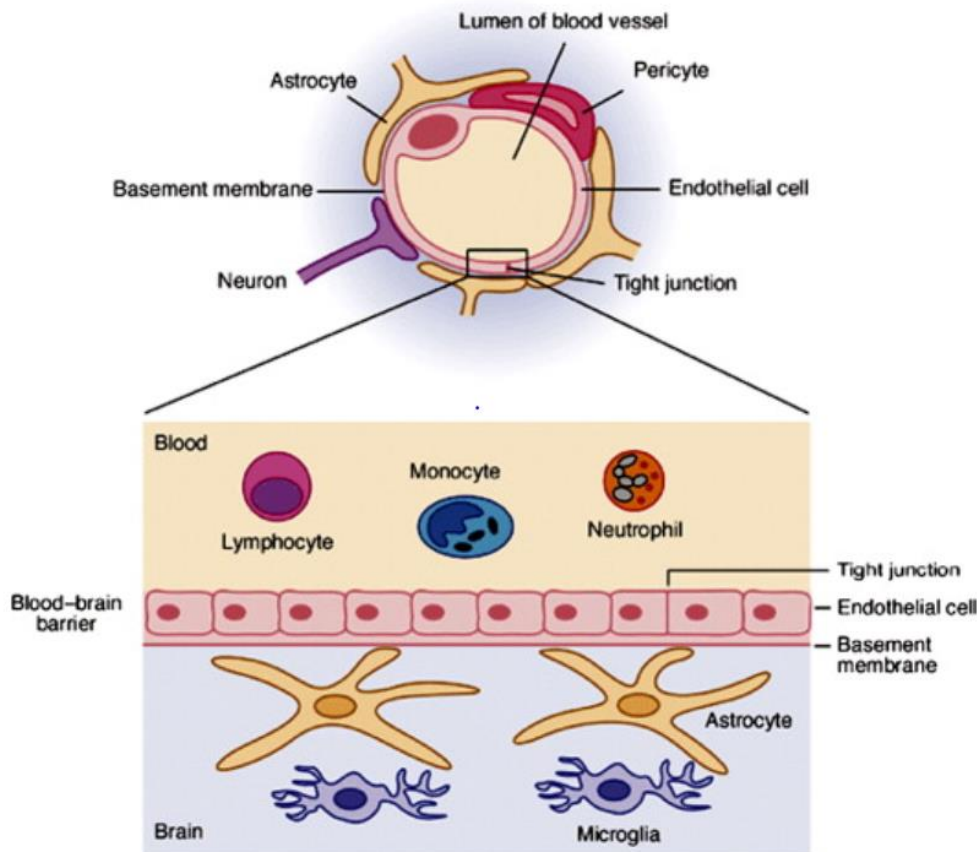


Figure 1-2 A representative cross-section of a cerebral capillary of the BBB, taken from (45)

Though the BBB may be disrupted in the presence of inflammation and tumours, the extent of disruption is variable and this makes it difficult to exploit for treatment purposes (33). The existence of these obstacles have implications for the successful treatment of brain tumours and other central nervous systems (CNS) conditions. Some of the approaches to overcome the obstacles, especially the presence and action of the BBB, and to improve clinical translation of chemotherapy include disruption of the BBB, modification of drug properties to suit entry into the BBB, or conjugation of ligands to the drug or drug carriers which facilitate

transport across the BBB (46,47). This project looks to develop a nanoparticle delivery system that will avoid the need to cross BBB by applying the delivery system locally to residual tumours within the brain post-surgery.

1.4 Nanoparticulate delivery system

1.4.1 Case for nanoparticles

The concept of nanotechnology originated in the 1950s and has now grown in leaps and bounds (48). It developed from the need to reconstruct, treat, repair and interact with the body at the molecular level (49). Though existing small molecule drugs also interact with different tumour and host tissues at the molecular level, nanoparticles offer the potential to alter their therapeutic efficacy and side effect profile (50). The idea of targeted delivery as a way of improving pharmacotherapy was first conceived in 1908, by Paul Ehrlich (51). Since then the formulation of medicines has grown from solutions, tablets, capsules and emulsions to more specific delivery systems. Research into nanomedicine is aimed at developing medicines with improved specificity and maximum efficacy with minimal side effects.

Nanoparticles (NP) are colloidal-size particles with particle size typically less than 1 micrometre (52). These particles can be modified to achieve targeted delivery, improved solubility, altered biodistribution, and improved access through biological barriers (53). Micron-size particles delivery systems have been used as depot systems, because they, due their size, cannot penetrate through tissues in the body. Microparticles circulating in the bloodstream might block capillaries and cause an embolism (54) and will be quickly removed and accumulate in the liver and spleen (55).

Particles in the small end of the nano-size range can bypass some physiological barriers (56). The particle size, the presence of an adsorbed hydrophilic material such as PEG, the zeta-potential and hydrophobicity all control the clearance, retention time in the blood, transport across and penetration into membranes, cellular uptake, drug accumulation and release at target site (57).

1.4.1.1 Applications of Nanoparticles

Nanoparticles can be used to achieve both control the release of encapsulated drug and can be functionalised with a target-specific ligand, to optimize their biodistribution towards the target site. In this way they can optimise the therapeutic efficacy and dosing of incorporated drugs (58). Targeted and controlled drug release to achieve local delivery of drugs is especially important in the treatment of diseases like cancer where success depends on the ability to treat tumour and spare other tissues.

Nanoparticles are also used in diagnostic imaging and regenerative medicine where normal human cells can be replaced or engineered to re-establish regular function (59,60). Furthermore, the delivery of problematic drugs can be enhanced with nanoparticles, for instance, due to their ability to help overcome problems like poor water solubility (61), erratic and unpredictable drug release and toxicity. The routes of administration used to deliver NP include parenteral (62), ocular (63), nasal (64), intra CSF, interstitial, regional arterial, etc.

The delivery of nanocarriers into specific target sites in the body, which is an important advantage of NPDS, can be either passive or active. Passive targeting relies on the localization of nanoparticles based on pathophysiological properties of the target site. An example of this is the Enhanced Permeability and Retention (EPR) effect. This refers to the

situation that some tumours have a 'leaky vasculature' which aids passive extravasation of material from systemic circulation into tumours and, due to defective lymphatic drainage which inhibits clearance from the extracellular space, resulting in its accumulation in tumour (65).

Active targeting normally refers to localization that occurs *via* interaction of nanoparticles surface modified with specific targeting moieties and receptors that are specific for target tissues. Molecules like antibodies (66), carbohydrates (67), nucleic acid (68) are attached to nanoparticles as targeting or affinity moieties. The attached moieties allow the NPDS to preferentially accumulate at specific cells/tissues possessing particular receptors or antigens (69). The ability to do this depends on the binding properties of the cell receptors to the ligands present on the surface of the nanoparticles (70).

This strategy offers benefits such as improved tumour accumulation and side effects reduction, due to the enhanced ability of the functionalised nanocarriers to accumulate preferentially at the target sites in comparison to that at non targeted sites (71). This strategy is advantageous if the cells possess the target receptors, however, in tumours which are heterogeneous, with potentially heterogeneous cell surface receptors, the benefit of the strategy will be limited because drug accumulation would not occur in the cells not expressing the targeted receptor.

The use of nanoparticles in the treatment of diseases like cancer has been justified mainly because of their ability to cross biological barriers and also their ability to protect their payload and deliver it intact to the target site. For CNS delivery, nanoparticles can be used to deliver drugs that usually are unable to penetrate the BBB (72). Active targeting is used to dual functionalise nanoparticles that both cross the BBB and target tumours (71). They can, therefore, be used to improve treatment of diseases via a

more efficient delivery to target sites. Examples of such that have been used for delivery to the brain are shown in Table 1-3.

Drug	Polymer	Surface modification	Size (nm)
Aclarubin	Cationized albumin	Maleimide-PEG	50-58
BCNU	PLA	Transferrin	120
Camptothecin	Poly ξ caprolactone- PEG (PCA-PEG)	PEG	130-280
Doxorubicin	Poly(butyl cyanoacrylate) PBCA	PS80	270
Etoposide	Tripalmitin	No modification	362, 391
Gemcitabine	PBCA	Polysorbate 80 (PS80)	112
Methotrexate	PBCA	PS80	87 - 318
Paclitaxel	PCA-PEG	angiopep	90
Temozolomide	PBCA	PS80	136

Table 1-3 Some examples of drugs bound to nanoparticles for delivery to the brain, taken from (31,73).

1.4.1.2 Types of Nanoparticles

The different types of nanocarriers include polymer conjugates, carbon nanotubes, gold nanoparticles, liposomes, micelles, dendrimers and polymeric nanoparticles (Figure 1-3) (74). Liposomes were the first generation of NP. These are made up of lipid bilayers surrounding an inner aqueous core (75). Solid lipid nanoparticles are a lipid NP in which the drug is dispersed or dissolved in a hydrophobic lipid core (76). Dendrimers are a type of polymeric systems which possesses tree-like structure and are made up of a central core with two identical chemical functionalities (77).

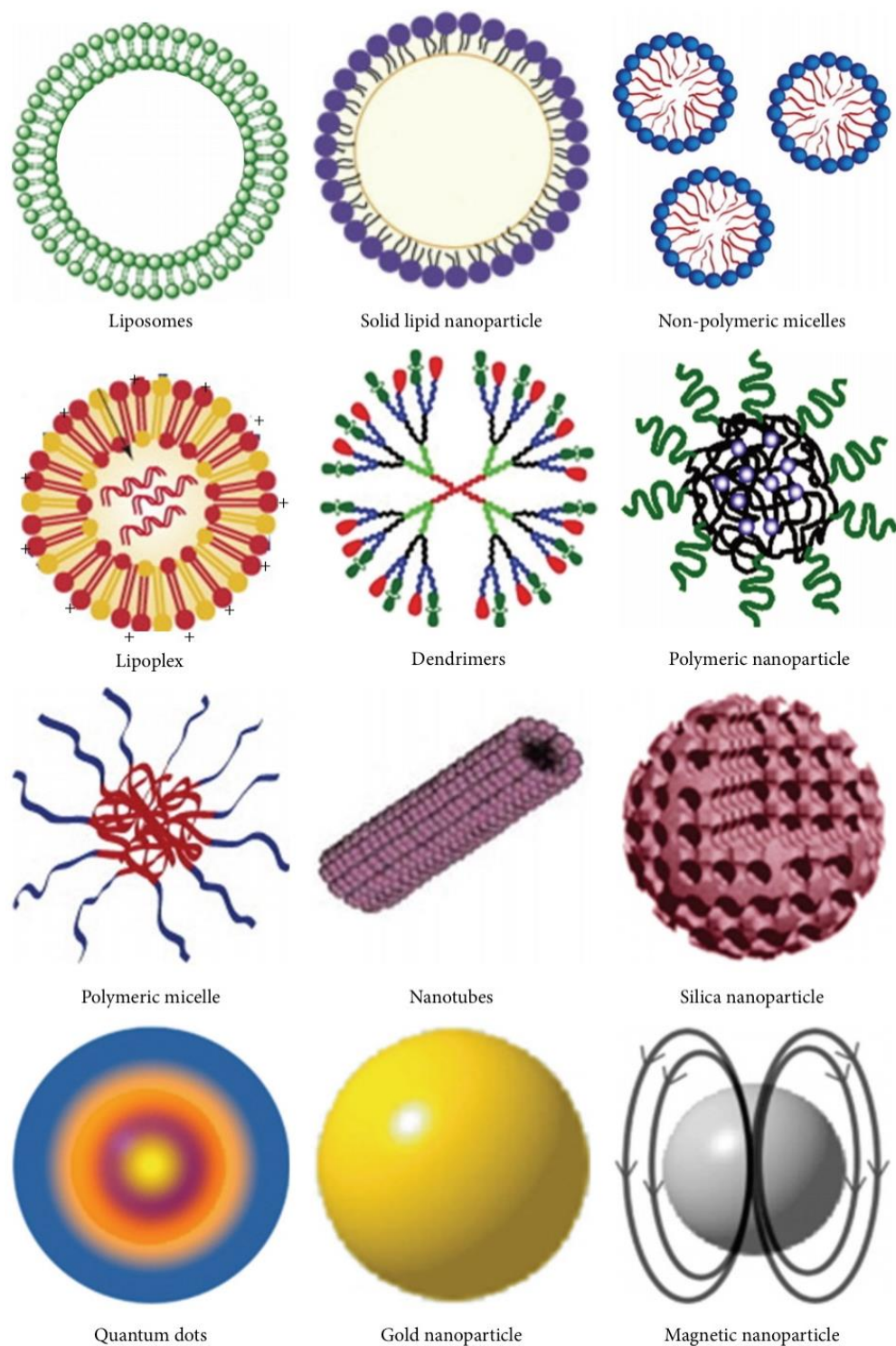


Figure 1-3 Types of nanocarriers used in cancer therapy such as polymer conjugates, carbon nanotubes, nanoshells, liposomes, micelles, dendrimers and polymeric nanoparticles picture from Massimo *et al* (72).

1.4.2 Polymeric nanoparticles

An ideal drug delivery system requires a carrier which conveys the drug to its target site. Polymeric nanoparticles are preferred for the delivery of drugs because they are stable and relatively easy and cheap to prepare (78). Polymeric NPs are made up of a polymer matrix in which the drug is either enclosed in a vesicular structure, termed nanocapsule, or dispersed within a carrier matrix, termed nanosphere.

The polymer is a key part of the nanoparticle and an ideal polymeric material would be nontoxic, biocompatible and biodegradable (79). The drug entrapment and release behaviour, biodegradation and drug release mechanism depends on the architecture of the polymer. Polymeric material could also affect the biodistribution of the loaded drug through its effect on nanoparticles surface properties, such as charge and hydrophilicity (15). Polymeric materials can be natural or synthetic, however, the synthetic polymers are more amenable to chemical, structural, and mechanical modifications that do not lead to major differences between batches (80).

The various polymeric materials that have been used include the biodegradable polyesters like poly(lactic acid), poly (glycolic acid) and their copolymer poly(lactic co-glycolide) (PLGA). Others include the poly alkyl cyanoacrylates, polyanhydrides, polycaprolactone (PCL). Important properties of these polymers that make them useful for nanoparticulate drug delivery include biodegradability through clearance by the body's metabolism, low-antigenicity and biocompatibility (79,80).

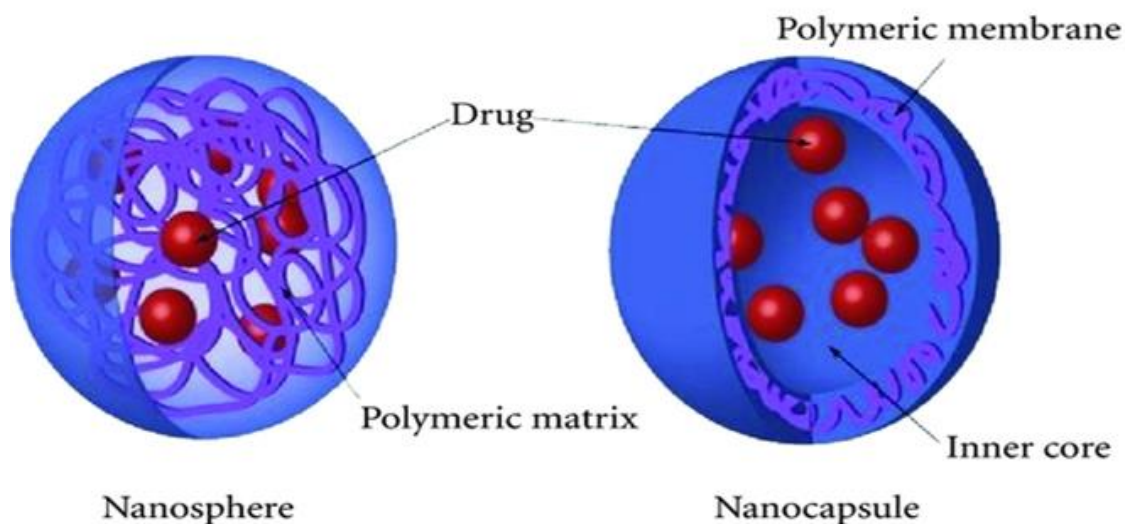


Figure 1-4 Two main structures of nanoparticles: Nanosphere, where the drug is entrapped within the polymer matrix and Nanocapsule where the drug is surrounded by the polymeric membrane, taken from (81).

1.4.2.1 Drug-polymer interactions

The conventional view in the literature is that hydrophobicity of drug and polymer drives drug incorporation and release. However, in recent literature, a more complex picture of drug-polymer interaction is emerging. There is some interest in the role played by the interactions between drugs and polymers in the entrapment of drugs into polymers. Drug-polymer interactions can be non-covalent like electro-static interactions, hydrogen bonding, van der Waals forces or π - π interactions and can be reversible. Chemical interactions, on the other hand, require bonding which involves ion exchange, protonation and complexation, and is usually irreversible. The type and extent of interactions that take place between drug and polymer are influenced by certain physicochemical properties like hydrophobicity of the drug and polymer, functional groups present in drug and polymer.

Molecular interactions between drug molecules and polymer may lead to reorientation of their molecules or their immediate chemical medium (82). These changes can be monitored using techniques such as ATR-FTIR (83) and contact angle measurements (84). Puncochova *et al* observed a stronger absorption of water by solid dispersions of Eudragit in combination with Valsartan, which normally is not seen in pure drug and polymer separately (82). The observed stronger interaction with water by valsartan-Eudragit dispersion was posited to be due to exposed functional groups as a result of structural changes. Changes like these were measured in this present work using contact angle measurements to measure the wettability behaviour of drug-polymer films compared to pure drug and polymer.

The method of nanoparticle preparation to be used in this present work is nanoprecipitation. In nanoprecipitation, from miscible organic solvent – water system, where a polymeric material is originally dissolved in organic solvent, a mixing of solvent and non-solvent (water) leads to polymer aggregation (85). Drug molecules present diffuse in the solvent mixture and direction of their diffusion would be driven by the affinity of the drug molecules interaction for the polymer species. The type of attractive interactions that the drug is able to have with polymers at this point is expected to drive drug entrapment (86). Also, a balanced hydrophobicity and hydrophilicity of the polymers has been suggested to play a role in drug encapsulation (86).

The diffusion of a drug through the polymer during drug release also depends on their affinity for one another. The higher the affinity by way of interactions the more the possibility of drug retention by the polymer. Sawant *et al* demonstrated the role of logP of the drug and the interactions between drug and polymer in drug release from polymers (87). They observed from their FTIR data that the attractive interaction between

hydroxypropyl cellulose (HPC) (hydrophilic polymer) and lidocaine (lipophilic drug) contributed to the slow release of lidocaine from HPC.

During nanoparticle preparation, the choice of polymer for a drug should be guided by the interactions between them. A screening method, which involves carefully selected analytical techniques may provide an opportunity for this. One of the methods used in this project involves monitoring the behaviour of specific drugs when placed in close proximity to different polymers with varying degree of substitution with side chains having different physicochemical properties. The addition of side chains to polymer backbones have been used to improve the ability of polymers to interact with drugs due to the presence of new functional groups hence possibly improving drug incorporation and release (88,89). For example, the incorporation of 5 fluorouracil into monomethoxy poly(ethylene glycol)-b-poly[γ -(carbamic acid benzyl ester)- ϵ -caprolactone] (mPEG45-b-PCABCLn) micelles was done by Yuan *et al* (90). They reported that conjugation of carbamic acid benzyl ester (CAB) groups to the PCL backbone improved the hydrophilicity of the polymers and consequently the drug loading and release.

Many of the commonly used polymers have limited potential for polymer-drug interactions because they have simple structures and limited opportunities for functional groups introduction that can interact with drugs. A polymer with a greater physicochemical variability would be a great advantage. The ability to make poly(glycerol adipate) (PGA) polymers with the OH groups substituted with side chains which confer different physicochemical properties and possibility of interacting in different ways makes it ideal for this project.

1.4.3 Polyglycerol adipate

The challenges of low drug incorporation and the need for more

controlled drug release has led to studies into the functionalisation of polymers. The approach of some studies is to have a modifiable polymer backbone which can be functionalised to **react ??** with the drug (91). A common strategy is to prepare pre-functionalised monomers with a protected functional group. The main disadvantage of this method is the low polymer yield obtained despite the lengthy process of step polymerisation and the use of many protecting groups (92).

The disadvantages of the step polymerisation method have led to the preparation of functionalised aliphatic polyesters using mild, chemo- or biocatalytic routes (92). For example, Crucho and Barros prepared sucrose and cholic acid-modified PLGA (93). Their work shows the potential of a modified PLGA based polymer as a vehicle for hydrophobic drugs. Kallinteri *et al*, synthesized polyglycerol adipate (PGA) to overcome some of these problems (78). Its synthesis is a one-step process that does not require a post-polymerization deprotection step (94).

PGA is a simple and versatile water-insoluble polyester that is biodegradable and synthesised by the action of the enzyme lipase, an enzyme derived from *Candida antartica* in the presence of tetrahydrofuran (THF) (Figure 1-6) (95). The enzyme lipase is selective for the primary hydroxyl group (OH), this leads to a polymer backbone with a free pendant OH group on every monomer unit. An advantage of a polymer such as PGA synthesised enzymatically is the clearance of such polymers via enzymatic degradation in the body (94). Naturally occurring enzymes in the lysosomes have been shown to be responsible for the rapid hydrolysis of PGA (96).

PGA is made up of monomers glycerol and adipic acid which are FDA approved to be safe (97). The monomers are linked by an ester bond in the backbone (Figure 1-5).

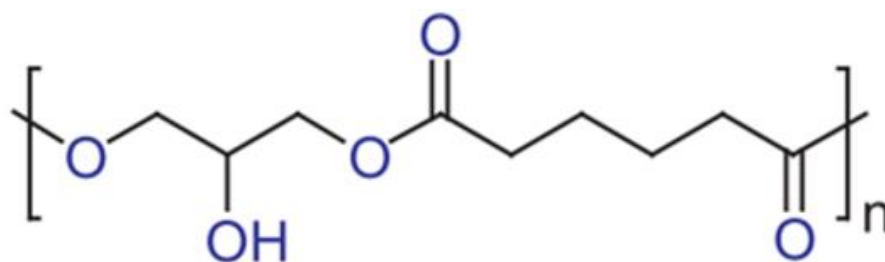


Figure 1-5 Structure of poly(glycerol) adipate with pendant hydroxyl group which can be substituted.

The free hydroxyl group can be substituted and modified with amino acids and acyl groups with various chain lengths and to varying extents from 0% to 100% to yield a polymer with new physicochemical properties. PGA based delivery systems can be tailored to meet the delivery requirements of different drugs (98). Taresco *et al* reported changes in the estimated logP and water contact angle of the PGA polymer as substitution with varying percentages of phenylalanine and tryptophan (94). A marked change in drug loading was reported by Puri when substitution was done using C8 acyl group (99). The drug loading of dexamethasone recorded was 4%^{w/w}, 8%^{w/w}, and 10.5%^{w/w} for 20%, 40% and 100% C8 substitution respectively. Kallinteri *et al* also reported varying successes in the amounts of dexamethasone incorporated in substituted PGA molecules in the absence of surfactants (78).

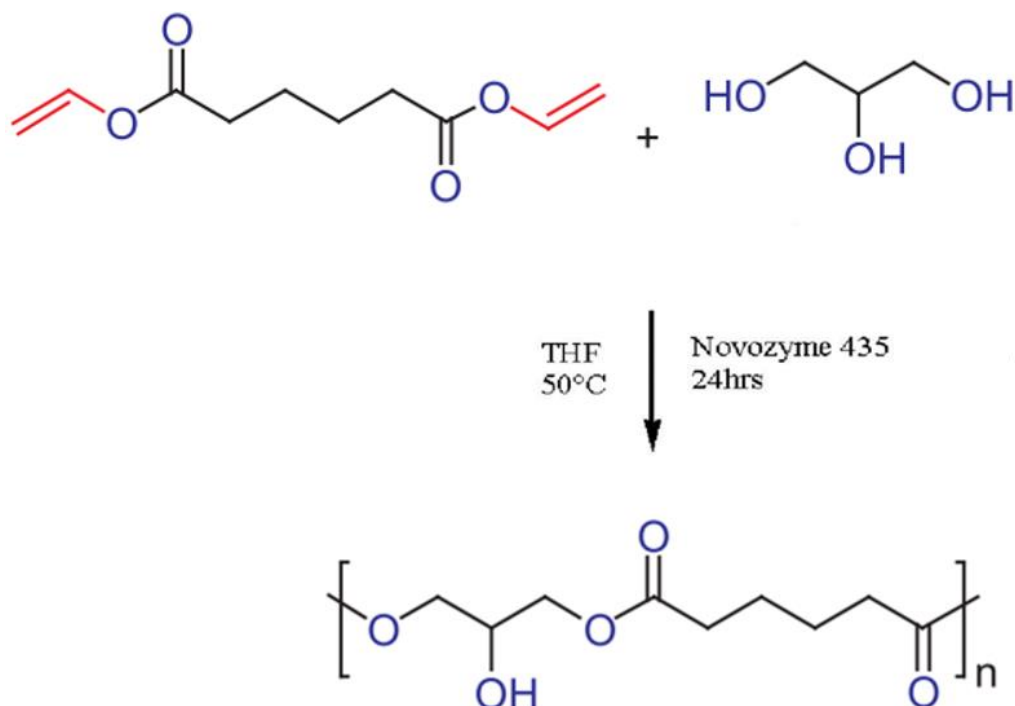


Figure 1-6 The synthesis of poly(glycerol adipate). Lipase catalysed synthesis with a preference for primary hydroxyl group

From Puri's and Kallinteri's works mentioned above, the physicochemical properties of PGA changes with the substitution of its free hydroxyl group, the nature of the substituent and the extent of substitution and these all play a role in the properties of the polymers made (94). This has led to changes in drug loading, particle size and encapsulation efficiency. The potential benefits of the advantages of PGA as an amendable polymer will be explored in this project.

1.4.4 Preparation of Nanoparticles

The preparation of nanoparticles has mainly been achieved by dispersing preformed polymers or polymerization of monomers.

1.4.4.1 Dispersion of Preformed polymers

Nanoprecipitation, emulsification/solvent diffusion and salting-out methods all fall under this category. The method of choice on this project is nanoprecipitation and will be discussed below.

1.4.4.1.1 Nanoprecipitation

This method, known as "interfacial polymer deposition or solvent displacement method" was first reported by Fessi *et al* (102). It is one of the most commonly used methods because it is simple, easily scalable, reduces the need for large amounts of toxic solvents and is reproducible (103). Nanoprecipitation is based on the precipitation of a substance when transferred from solvent to non-solvent phase. A water-miscible organic solvent containing a dissolved polymer when added slowly into an aqueous phase will spontaneously diffuse into the aqueous phase (Figure 1-7) (105). The polymer precipitates and solidifies due to the rapid diffusion of the solvent in the non-solvent, leading to the formation of droplets and the organic solvent is removed by evaporation. The removal of the organic solvent is done by evaporation at ambient temperature or with a rotavapor, leaving the nanoparticles (NPs) suspension in water (103).

The water/solvent ratio and the concentration of polymer dissolved in the organic solvent are examples of parameters that can be varied to achieve smaller mean particle size and a low polydispersity index (107).

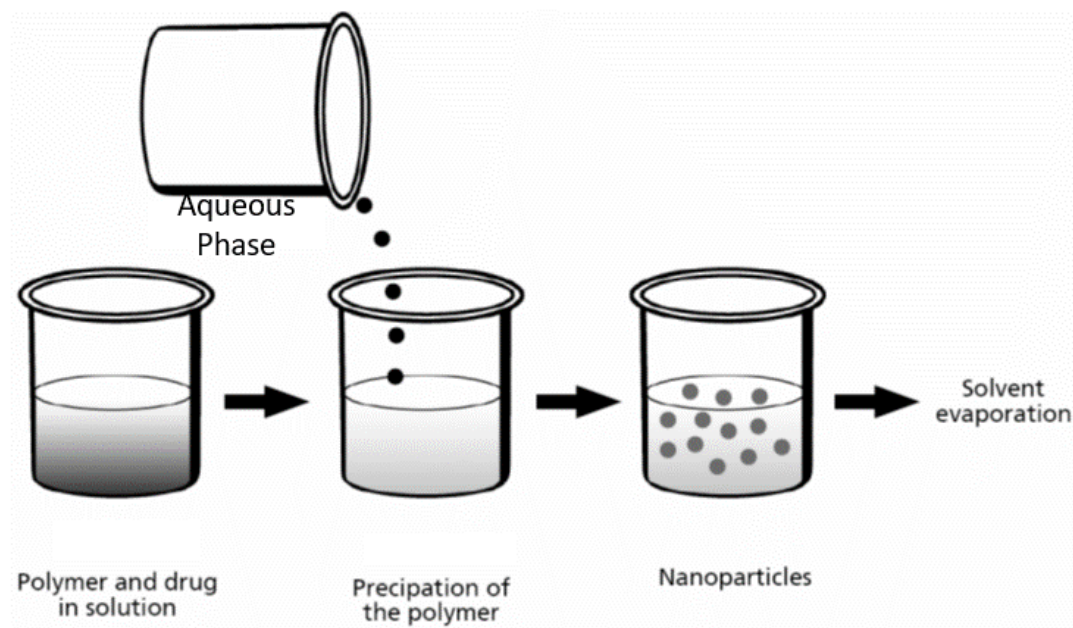


Figure 1-7 Illustration of Nanoprecipitation: Spontaneous diffusion of a water-miscible solvent containing the polymer upon its slow addition to the aqueous phase leading to the precipitation of the polymer (108).

Nanoprecipitation has been used for hydrophilic and hydrophobic drugs with the former being plagued with a number of difficulties especially in the area of drug encapsulation. Govender *et al*, reported low encapsulation with the incorporation of hydrophilic drugs using nanoprecipitation (109). This has been explained to be as a result of the hydrophilic drug diffusing into the aqueous phase. An important factor that also affects encapsulation efficiency is the rate of polymer precipitation. Mackenzie *et al* explained using computational modelling that good encapsulation depends on the surface activity of the drug and interaction of the drug with polymer chains (86).

After preparation, characterization is a step to determine the properties of the nanoparticles.

1.4.5 Characterization of nanoparticles

After preparation, characterization is a step to determine the properties of the nanoparticles.

1.4.5.1 Drug loading

Drug loading is an important parameter that shows the amount of drug incorporated into a nanoparticle. An ideal drug delivery system will deliver an appropriate amount of drug at appropriate rate to the target site. Hence, it is important that adequate drug loading levels are achieved in the NPDS. Though there is no general drug loading requirement, a NPDS must achieve a sufficient level of drug loading, which depends on the dose required to achieve the intended therapeutic effect for any drug (134). The problem of insufficient drug loading in nanoparticles cannot be compensated for by administering a larger dose of nanoparticles. This is because the endocytotic process of nanoparticle uptake is not infinite, it is pertinent to ensure that the limited amount taken up is delivering a sufficient amount of drug.

Drug loading is calculated in relation to overall polymer content (135). The expression of the percentage of drug entrapped in a nanoparticle is sometimes expressed as encapsulation efficiency, which does not reflect exact drug content rather it is only used as a tool to reflect the extent of drug wastage (117). Though encapsulation efficiency is commonly stated and accepted, it can sometimes lead to overestimation of actual drug content in the nanoparticles.

Actual drug loading can be determined by direct and indirect means. The indirect method requires that the amount of unentrapped drug is determined and then used to estimate the amount of drug encapsulated by difference. This method assumes that the rest of the drug is all

encapsulated which might not be so. Some drug loss might have occurred during the process and so is considerably less reliable and accurate. The direct method involves measuring the actual mass of the drug loaded in the matrix. This can be done by dissolving the drug-loaded nanoparticles and determining the drug content via standard analytical methods like HPLC or spectrophotometry. This method was chosen for this project.

The percentage drug loading was determined using the equation below:

$$\% \text{ Drug loading} = \frac{\text{Mass of drug entrapped}}{\text{Total mass of nanoparticles}} \times 100 \quad \text{Equation 10}$$

Where the weight of drug entrapped is the weight of drug dissolved in the nanoparticles. The total mass of nanoparticles is the weight of freeze-dried nanoparticles with the drug.

1.4.5.2 Drug release from polymeric nanoparticles

A NPDS can alter the safety, efficacy and toxicity profile of a drug if the drug remains loaded in it for an appropriate period of time (110). The mechanism of drug release, the *in vivo* biodegradation rate, diffusion coefficient and the *in vivo* environment all affect the rate of drug release from a NPDS (99). The release of drug from a delivery system is an important factor that contributes to the success of the system. This depends on its ability to deliver the incorporated drug in a purposeful and timely manner to its intended target site. It is important to retain the drug until it reaches the target site, this ensures that unintended drug losses are avoided and effective dose of the drug get to the target site.

Drug release precedes its action at target site however, its effectiveness depends on the ability of the drug to be released at the target site and the effectiveness of the process. The process of drug release from a modified DS may involve osmosis, dissolution, partitioning, diffusion, swelling and

erosion (111). The release of drug from the NP can be through swelling and diffusion through the polymer matrix, surface or bulk erosion (112) or desorption of drug bound to the surface. It can also occur by diffusion through a layer of polymer coating surrounding a drug core (113). Drug release can also be by enzymatic degradation of the polymer. The release of hydrophilic drugs from a matrix occurs by diffusion (111). The hydrophobic drug is expected to be released by swelling and or the erosion of the matrix. While this is what is expected, the release behaviour of drugs is not driven by hydrophobicity or hydrophilicity alone. The release behaviour of different drugs depends on other factors like the functional groups present, constituents of the delivery system, morphology and size of the NP, Physicochemical factors and expected site of action (for example the physiology of the CSF).

1.4.5.2.1 Monitoring Drug Release from Nanoparticles

Drug release profile can help in forecasting the potential of the nano-sized systems to achieve the intended therapeutic effect. In biological systems, there may be a number of factors which may additionally affect drug release, but the routine determination of drug release is the determination of the physicochemical factors giving a baseline level of drug release (more importantly retention). These *in vitro* tests may be used for ranking different nanoparticle formulations and to establish how long the carrier can hold on to the drug. Some of the factors that affect *in vitro* drug release behaviour include the drug concentration, physicochemical characteristics of the active substance, the nature, degradability, molecular weight and concentration of the polymer; the size of the nanoparticle, the conditions of the *in vitro* test medium (medium pH, temperature, contact time, among others) (114). The experiments should be carried out at physiological conditions where possible. Perfect sink conditions must be maintained, this means that the volume of the release medium must be at least three to ten times that which is required to make

a saturated solution of the drug (116). This ensures that the concentration of the drug in the release medium is so low that it does not influence the rate of drug release.

The determination of drug release for disperse systems, for example, nanoparticles, faces a lot of challenges due to their small size (115). Methods such as dialysis (117), centrifugal ultra-filtration (118) and ultracentrifugation (119) have been used to separate the drug from the carrier. The use of ultracentrifugation is limited due to the need for longer centrifugation times and the possibility of leakage of drug from smaller particles or in systems where the disperse and continuous phases have similar densities. The filtration method may cause sedimentation and drug losses could occur due to repeated washing. For centrifugal ultrafiltration, which involves the use of some force to filter the sample through a membrane, the limitation here is from the force of filtration and centrifugation. This can lead to compression of the particles leading to an overestimation of the rate of drug release (120). Drug losses can also occur during filtration which has to be accounted for. The above listed methods may also have a small amount of nanoparticles getting into the filtrate or supernatant which could lead to error in the release results (121).

Dialysis involves the separation of a drug from the carrier using a dialysis membrane of the right molecular weight cut off. The dialysis membrane is permeable to the released drug and serves to separate the nanoparticles from the free drug (56). The process involves periodic sampling of an outer medium to determine drug content.

The release of drug from the carrier is governed by the following processes (101).

1. The diffusion of the drug through the solid matrix of the carrier.
2. The solvent penetrates the nanoparticles and dissolves the drug. The dissolved drug then diffuses out in solution.
3. The free drug freely diffuses through the dialysis membrane.

Some of the key limitations of the dialysis technique include the ability to maintain sink condition in the donor compartment (115). Washington observed the influence of the partitioning of the drug between the carrier and the aqueous phase in the donor compartment (122). As sampling is done from the outer compartment, the rate of drug release from the carrier has to be accounted for. The interpretation of the results needs to consider this lag. In addition to this, there is a possibility of interaction between the released drug and the carrier.

On this project, the chosen method was dialysis and possible drug adsorption to the carrier was accounted for by using a control where empty carrier was spiked with free drug solution. The addition of a surfactant to the dissolution media was done to ensure sink condition due to the low water solubility of two of the drugs (etoposide and teniposide). The analysis of the results obtained from *in vitro* drug release studies helps us to determine what drives the release of drug from the polymer matrix.

1.4.5.2.2 Drug release models

The *in vitro* release of drugs from polymeric drug carriers has been described to consist of two phases: a first phase called “burst release” is known to be due to the adsorbed drugs on the surface or dispersed close to the surface or the carrier, and a second phase of slower release that is attributed to release of drug which is located dispersed within the polymeric matrix or the core. A study of the release kinetics of the drug from a delivery system (DS) can help formulation scientists to understand the mechanisms involved (111,112).

Drug release models are used to describe the release of drug from a controlled release system and the various mechanisms involved. The quantitative interpretation of the experimental values of drug release assays can be done through mathematical equations (111). The effect of parameters such as the composition, shape and size of the delivery system on the rate of drug release can be identified and further understood with mathematical modelling (123). Examples of the release kinetic models are zero-order, first-order, Baker-Lansdale, Higuchi, Hixson-Crowell, Gompertz and Korsmeyer-Peppas.

In zero-order kinetics, the drug release is a function of time. It is independent of the concentration of the drug and occurs at a constant rate. It is represented by the equation below:

$$Q_o - Q_t = K \cdot t \quad \text{Equation 1}$$

Where Q_o is the mass of the drug at time zero, Q_t is the remaining amount of drug at time t while K is the constant of proportionality. Expressed simply as:

$$f_1 = K_o t \quad \text{Equation 2}$$

Where f_1 is the cumulative amount of the dissolved drug at this time and K_o is the apparent velocity of dissolution and it is a constant. A plot of cumulative release against time results in a straight line whose slope is equal to K_o (Figure 1-8).

Zero-order is applied when constant drug release is expected from drug delivery systems such as transdermal systems, matrix tablets with low-soluble drugs and oral osmotic tablets (124–126).

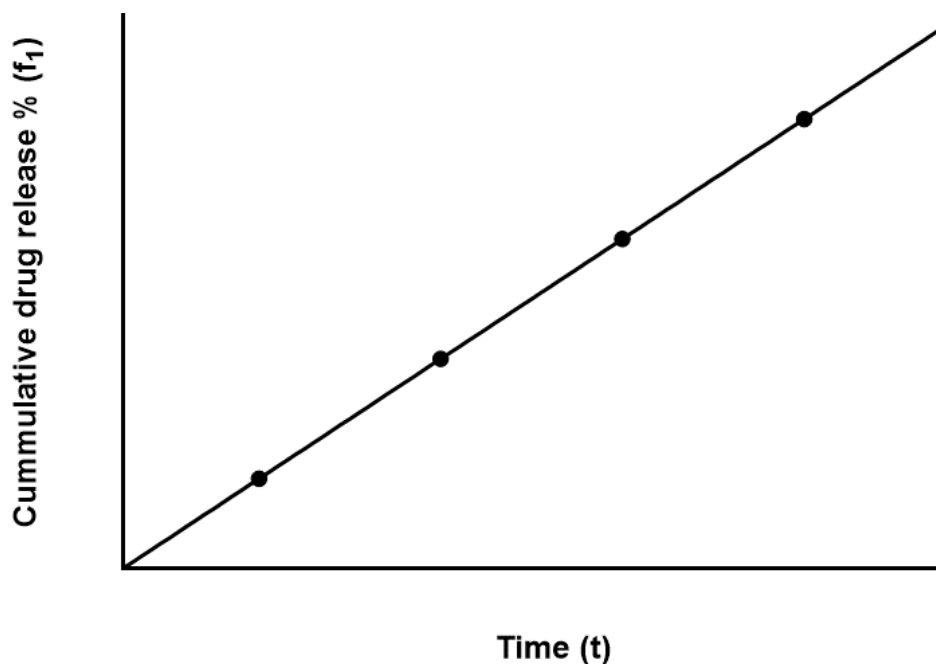


Figure 1-8 Zero-order kinetics represented graphically: y-axis (f₁) represents the cumulative release of active ingredient at time (t), the x-axis represents the time.

For first-order kinetics, the rate of release of the drug depends only on concentration. This is represented in the following equation:

$$\frac{dC}{dt} = -kC \quad \text{Equation 3}$$

Where C is the drug concentration and k is the first-order release constant (Figure 1-9 and Figure 1-10). First-order model is applied to the release of water soluble drugs in a porous matrix (111). The concentration of soluble drug released from a porous matrix is proportional to unreleased drug. Hence, first-order drug release is not constant and reduces with time.

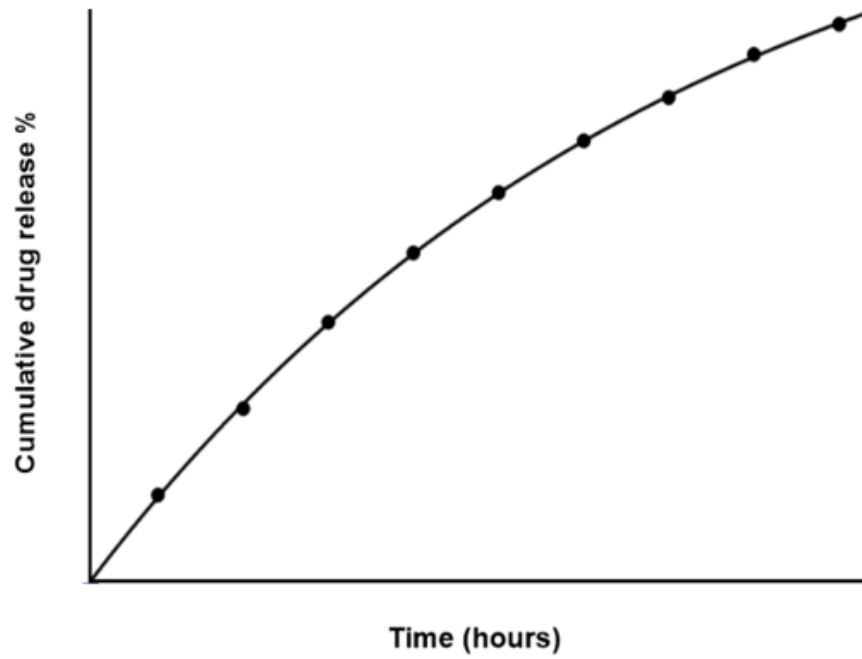


Figure 1-9 first-order model of drug release represented graphically: x-axis represents cumulative drug release (%) against time (minutes)

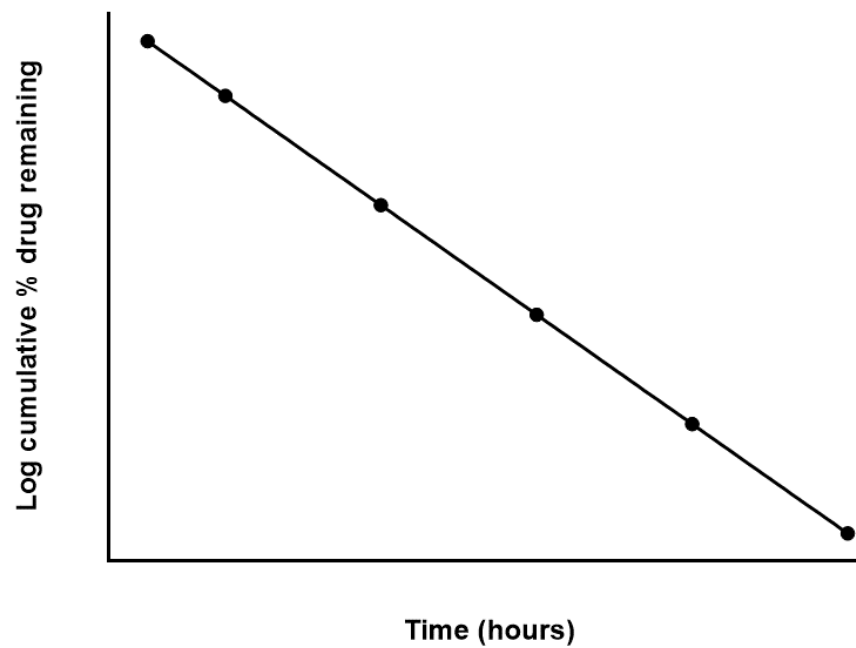


Figure 1-10 First order model of drug release represented graphically; log cumulative drug release (%) against time (minutes).

The Higuchi model is used to model the rate of drug release from matrix systems (127). This model states that the rate of drug release is proportional to the square root of time. Consider the following equation

$$Q = K_h \sqrt{t} \quad \text{Equation 4}$$

Where Q is the quantity of drug released in time (t), K_h is the Higuchi release constant. A graphical representation of this equation gives a linear graph whose slope is K_h (Figure 1-11). Drug release can be interpreted to be diffusion controlled if a higher correlation coefficient is obtained from the Higuchi plot.

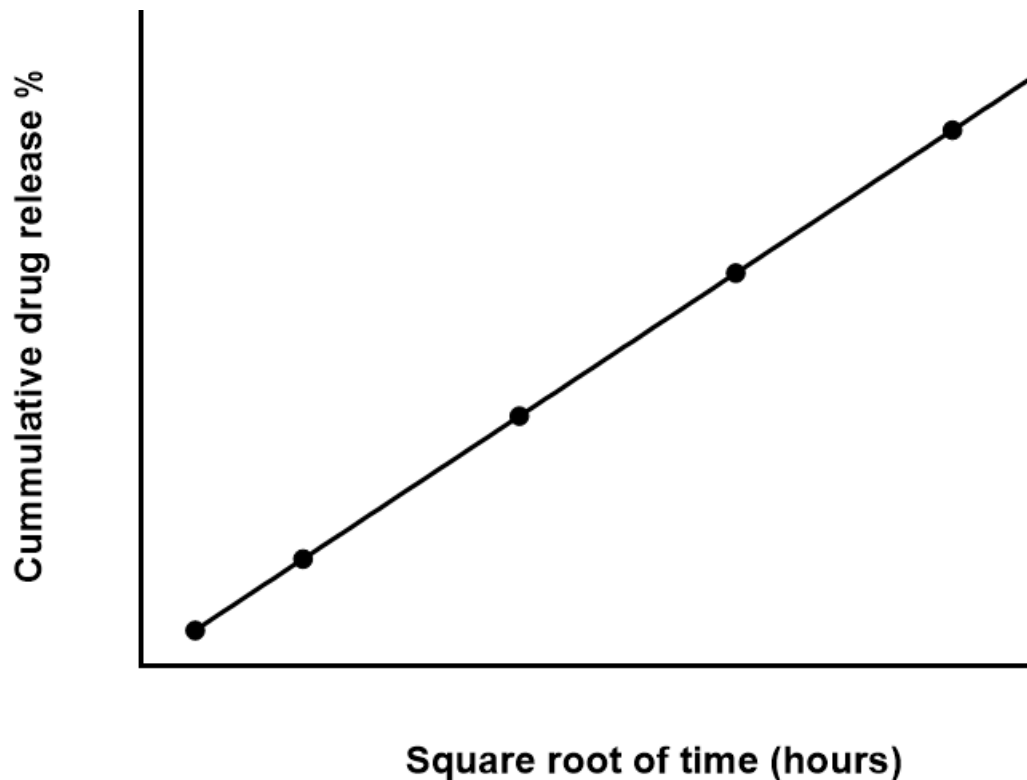


Figure 1-11 Higuchi model represented graphically: cumulative drug released % versus square root of time.

The Higuchi model is based on the following assumptions (128,129).

1. The solubility of the drug is lower than the starting drug concentration in the matrix.
2. The diffusion is in one direction only, the effect at the edges is negligible.
3. The size of the drug molecules is much lower than the thickness of the dosage form.
4. There is negligible swelling or matrix dissolution.
5. The drug diffusivity is constant, this is valid if the polymer does not swell significantly when it comes in contact with water.
6. There are perfect sink conditions in the release environment

The Higuchi model has led to the development of other release models such as the Korsmeyer Peppas, that can be applicable to some other dosage forms. The Korsmeyer–Peppas model also known as power law, allows the explanation of drug delivery by erosion and/or matrix dissolution (112). It is a generalized model of the Higuchi equation, it is used when the mechanism of drug release is unknown or more than one type of drug release mechanism is involved (130). This model is represented by the equation

$$\frac{M_t}{M_\infty} = kt^n \quad \text{Equation 4}$$

where M_t/M_∞ is the fraction of drug released at time t ; k is the release constant, n is the diffusion exponent that is representative of the release mechanism (131). The value of n enables the classification into Fickian or non Fickian models

If n equals 0.5, the equation is equivalent to the Higuchi release. While n between 0.5 and 1.0 is an anomalous release mechanism and n equal to 1 is zero order.

Zero-order, first-order, Higuchi and Korsmeyer Peppas were applied to the formulations made on this project because they are matrix systems, containing low soluble drugs (zero-order) and they are systems with unknown mechanism of drug release (Korsmeyer Peppas).

Hixson Crowell model is used to describe the release of drug by dissolution from tablets or particles with changes in their surface area and diameter (111). This model is used mainly to describe a dosage form that is spherical.

$$\sqrt[3]{Q_0} - \sqrt[3]{Q_t} = K_{HC} \cdot t \quad \text{Equation 6}$$

Where Q_0 is the initial amount of drug, Q_t is the cumulative amount of drug release at time t , K_{HC} is the Hixson Crowell release constant and t is time in hours (126). The plot is expected to be linear if the shape of the dosage form reduces proportionally with time (132). This model was not applied on this project because the particles prepared are not expected to degrade and reduce in dimension proportionally.

Baker Lansdale model is derived from the Higuchi model and is used to linearize the data obtained from release studies of microcapsules or microspheres (132,133).

$$\frac{3}{2} \left[1 - \left(1 - \frac{M_t}{M_\infty} \right)^{2/3} \right] \frac{M_t}{M_\infty} = \frac{3D_m C_{ms}}{r_0 C_p} - t \quad \text{Equation 7}$$

M_t is the amount of drug released at time t , M_∞ is the amount released at the infinite time, D_m is the diffusion coefficient, C_{ms} is the solubility of the drug in the matrix, r_0 is the radius of the spherical matrix, and C_0 is the initial concentration of the drug in the matrix (111). The particles made on this project are not microspheres and microcapsules so this model was excluded.

The Gompertz model is also used to described the dissolution of a dosage form and is expressed by the following equation (132):

$$X(t) = X_{max} \exp [-\alpha e^{\beta \log t}] \quad \text{Equation 9}$$

X(t) is the percentage of drug dissolved at time t divided by 100, X_{max} is the maximum dissolution, α determines the undissolved proportion of drug at time t=1 and β is the dissolution rate per unit of time described as shape parameter (130). This model was excluded because it is mainly applied to dosage forms containing drugs that have good solubility and intermediate release rate (132) and all the drugs used in this project do not fit this description.

1.4.5.3 Particle size and zeta potential

The advantages associated with the use of nanoparticles in drug delivery are mainly due to their size. The particle size of a nanoparticle impacts its *in vivo* behaviour such as uptake and clearance (136). Smaller particles are able to avoid the mononuclear phagocytic system and accumulate more at target sites (137). A size range of 70-200 nm is required for prolonged systemic circulation and with surface modified nanoparticles (138). The enhanced permeability and retention effect posited to be responsible for passive targeting also depends on the size (76). The disorganised nature of tumour epithelium allows leakage of macromolecules and nanoparticles up to 500 nm in size into the interstitial space of the tumours. Due to the absence of lymphatic vessels in many tumours, particles (139).

Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) or dynamic light scattering (DLS) are the techniques used to determine the size and surface morphology of NP. Key considerations when picking a technique includes, the polydispersity of the particles, the tendency of the particles to aggregate. Domingos *et al* observed the

microscopic and the light scattering techniques offered unique advantages and disadvantages based on the differences in the results obtained as conditions such as the concentration, polydispersity and complexity of the particles changed (140).

DLS also known as photon correlation spectroscopy (PCS) or quasi-elastic light scattering can be used to determine the size of sub-micron particles. DLS is ideal for particles from 0.3 nm to several micrometres. It determines hydrodynamic size distribution by measuring the amount of light scattered by dispersed colloidal particles under Brownian motion (141). The particle size obtained differs from that obtained using techniques such as electron microscopy.

The intensity of light scattering is measured at a fixed angle and the fluctuations that occur due to Brownian motion reflects the rate of diffusion of its particles (136). The diameter measured here being the hydrodynamic diameter indicates how a particle diffuses within a fluid. This is determined from the Stokes-Einstein equation (142):

$$d(H) = \frac{kT}{3\pi\eta D} \quad \text{Equation 11}$$

Where $d(H)$ is the hydrodynamic diameter, D is translational diffusion coefficient, k is Boltzmann's constant, T is absolute temperature and η is the viscosity of the dispersion. The particle is assumed to be spherical in shape. The reported particle size is that of a sphere with the same translational diffusion coefficient and scatters light in a similar way as the particle being measured. Multiple scattering, where the scattered light interacts with other particles in the dispersion before reaching the detector leading to a reduction of intensity, might occur if the dispersion is too concentrated. This can be avoided by diluting the sample to an optimum concentration (143). Another limitation of this technique may occur if very large particles are present in the dispersion. A plot of the

relative intensity of light scattered by particles of different diameters is the size distribution obtained, known as the intensity distribution. According to the Rayleigh scattering theory, the intensity of the scattered light (I) is directly proportional to the radius raised to the sixth power (143). The presence of larger particles causes a skew in the intensity distribution. For instance, a 50 nm particle scatters light up to one million times larger than a 5 nm particle. A number or volume distribution is useful when large particles are present in order to assess the relative importance of these populations.

Zeta potential denotes the electrokinetic potential of dispersed particles in suspension at the shear plane of a particle moving under an electric field. A charged particle in a dispersion develops an electrical double layer on its surface. The Electrical Double Layer (EDL) is made up of the same ions as the particles and also the opposite ions from the medium of the stern layer. The stern layer is an inner layer which contains ions opposite in charge to the particles (144). The application of an electric field causes the particle and the Stern layer and a certain part of the EDL to move towards the electrode that is oppositely charged (electrophoresis). As electrophoresis occurs, a hypothetical plane (slipping plane) which is the EDL acts as the interface between the moving particle and the layer of the dispersant surrounding it.

Zeta potential is the potential difference at the interface and it is measured, in this study, by an instrument based on electrophoretic light scattering (ELS). ELS is a technique that combines electrophoresis with light scattering spectroscopy to determine the diffusion coefficient and the relative mass-weighted ratios of charged macromolecules in solution (145). It can be used to determine stability because the charged particles are less prone to aggregation as electrostatic repulsion forces keep them apart. Zeta potential influences biological behaviour like cell uptake and

toxicity. It is affected by the pH, type of ions and ionic strength of the environment in which the particle is present (141).

1.4.6 Challenges associated with the clinical translation of nanoparticle research

Research in nanomedicine has attracted attention because of the potential ability of nanoparticles to interact differently from drugs at the cellular level and their structural and functional advantages (80). The level of interest in the development of nanocarrier-based medicine has been phenomenal. However, there are still only a handful of such products on the market. Examples of such include Abraxane® (61) and Doxil®(146). Although the first nanoparticles for the delivery of vaccines and drugs were developed in the late 1960s (51), the first clinically approved nano-based product, Doxil®, was introduced in 1995. Since then, research has continued with about 77 currently in clinical trials and 51 food and drug administration (FDA) already approved nanoparticle formulations (147).

Table 1-4 shows some of the approved nanocarriers, applications and routes of administration and their approval dates. Recently, the production of Depocyt®, one of the few approved liposomal formulations has been discontinued due to problems with its manufacturing process (148).

Encapsulated drug	Trade name	Carrier system	Administration route	Application	Approval
Leuprolide	Lupron Depot [®]	Microparticles	Intramuscular	Analog of gonadotropin-releasing hormone	1989, USA
Amphotericin B	Ambisome [®]	Liposomes	Intravenous	Visceral leishmaniasis treatment	1990, Europe 1997, USA
Doxorubicin	Doxil [®]	Liposomes	Intravenous	Anticancer therapy	1995, USA 1996, Europe
Daunorubicin	DaunoXome [®]	Liposomes	Intramuscular	Anticancer therapy	1996, Europe 1996, USA
Cytarabine	Depocyt [®]	Liposomes	Intrathecal	Lymphomatous meningitis treatment	1999, USA
Doxorubicin	Myocet [®]	Liposomes	Intravenous	Anticancer therapy	2000, Europe
Verteporfin	Visudyne [®]	Liposomes	Intravenous	Photodynamic treatment of age-related macular degeneration	2000, USA
Morphine	DepoDUR [®]	Liposomes	Epidural	Analgesia	2004, USA
Vincristine	Onco TCS [®]	Liposomes	Intravenous	Anticancer therapy	2004, USA
Doxorubicin	Transdrug [®]	Nanoparticles	Hepatic intra-arterial	Hepatocellular carcinoma treatment	2005, USA
Paclitaxel	Abraxane [®]	Nanoparticles	Intravenous	Anticancer therapy	2005, USA 2008, Europe

Table 1-4 Examples of marketed nanocarriers, applications, and their approval dates in EU and US taken from (103)

This relative inability to transfer laboratory efforts to the industry is partly due to problems associated with drug loading (52,149), stability, leakage of the loaded drug, toxicity, inadequate understanding of the interaction between the polymer and drug (150). Specifically, low stability during storage and leakage of drugs are problems associated with the use of liposomes (151).

Despite, the low translation rate from bench to product, the use of nanoparticles in drug delivery still has great potential and holds the key to treating and preventing many diseases. Many researchers have shown that nanoparticles can improve the biodistribution of drug which can ultimately lead to better treatment outcomes. Erythrosine loaded PLGA nanoparticles were reported to have improved and prolonged antibacterial effect compared to unencapsulated erythrosine (152). Also, Mérian *et al* showed up to 10 fold increase in tumour accumulation of liposomal pentamidine compared to the unencapsulated drug (153).

The tremendous investment in the research on the use of nanoparticles has not yielded a commensurate improvement in cancer treatment. Though the few approved nanoparticle-based formulations have improved the side effect profile of the loaded drug, the overall impact on treatment outcome is unsatisfactory. As earlier stated, the erratic or burst release and low drug incorporation are a key hindrance to the efficient use of nanoparticles to deliver drugs. Other barriers will be discussed below.

1.4.6.1 Barriers to nanoparticle drug delivery

Despite the promise of improved drug targeting to the desired site by nanoparticles, less than 1% of the incorporated drugs have been delivered to solid tumours (154). These low drug concentration at the target site contributes to multidrug resistance, which is usually compensated for by the administration of higher drug doses. When nanoparticles are

administered systemically by parenteral route, they interact with blood components termed opsonins which adsorb onto the nanoparticles and enable clearance by the phagocytic action of the organs of the mononuclear phagocytic system (MPS) (50). This dramatically influences nanoparticles biodistribution and their ability to reach the target site.

Nanoparticles also have to overcome the physical barrier posed by the epithelium, the vascular endothelium and their underlying basement membrane. The tight junctions that exist between the endothelial cells and the underlying basement membrane exclude materials based on size (50). Larger fenestrations exist in organs like the liver, while the endothelium is discontinuous in organs like the spleen. These physiological barriers pose a size-dependent hindrance to the ability of nanoparticles to reach to their target sites (155). For example nanoparticles below 5 nm are filtered out by the kidney (57) and larger nanoparticles $\geq$ 250 nm, despite coating with a hydrophilic material which reduces opsonisation, are removed by the liver and spleen (138).

Nanoparticles may, however, capitalize on the leaky endothelium caused by the presence of inflammation and diseases like cancer. The endothelium becomes leaky and allows the passage of particles of larger size up to 700 nm in some capillaries (155). The EPR is based on improper regulation of angiogenesis (76). The absence of effective lymphatic vessels in tumours leads to a retention of the nanoparticles in the extracellular matrix leading to retention of the drugs (155). This allows the entry of the nanoparticles containing the drugs into the tumour cells (53).

Relying on passive targeting via the EPR for tumour accumulation has its limitations. Firstly, some tumours do not exhibit the features responsible for the EPR effect and also the vessels in a tumour may not be leaky throughout the tumour. Secondly, it is a complex process which is difficult to control and may vary from tumour to tumour thereby leading to poor

drug delivery and drug resistance (53). The understanding of the EPR effect is evolving and the many contributions of the heterogeneous tumour microenvironment need to be better understood for it to be more useful (123). Lastly, passive drug entry through a leaky blood brain barrier can make the CNS more penetrable. The dependence on this for drug delivery is unreliable because as the tumour load decreases and the blood brain barrier gets repaired drug entry declines and may eventually stop, leading to possible treatment failure.

In the tumours, the barriers affecting drug diffusion to the tumour core include the heterogeneous tumour vasculature, the composition and changes to the extracellular matrix, high interstitial fluid pressure, lack of convective flow and rapid drug clearance, especially from the CSF (32,156,157). These barriers limit the trans-vascular and interstitial transport of drugs and nanoparticles through the tumour microenvironment. In addition to this, the disorganised vasculature also limits drug diffusion across the tumours cells (158). The additional extracellular component of the tumour stroma and the unrestrained cell growth makes for a dense, tortuous cellular environment which further limits drug diffusion and contributing to the failure of treatment (159,160). Furthermore, some drug loss occurs due to the early release of drug while crossing the extracellular matrix.

1.5 Drug delivery to brain tumours: systemic vs local delivery

Firstly, in chemotherapy, the systemic route to the tumour which relies on the EPR effect is the most commonly used route to passively deliver drugs to tumours by nanoparticles and it has not been very successful (154). Drug delivery to the brain is further limited by the BBB. The whole-body distribution is usually compensated for by increasing the dose to achieve

maximum efficacy but leads to more side effects due to exposure of the cytotoxic agents to healthy cells in the body. The need to circumvent the BBB and avoid loss of drug via systemic route creates a case for novel strategies to improve the delivery of drug. Local delivery will ensure therapeutic doses of drugs is localised at the intended site while facilitating control of the exposure rate and time (161) helping to minimise off-target side effects (46). This may help to achieve better treatment outcomes especially regarding tumour reoccurrence and treatment of tumours left behind after resection (74). In some cases, local delivery can also help the BBB to be bypassed in some cases (162), this also increases the potential of the administered drug to get to the intended site in the brain.

The use of local delivery though desirable still requires further modification to ensure efficient delivery. To improve the outcome with post-surgical residual cancer cells, local delivery with improved selectivity is desirable. This project exploits the results from Meng *et al*, which demonstrated the preferential uptake of PGA NP by MB cells. She demonstrated that RBITC labelled PGA nanoparticles were taken up six times more by tumour spheroids compared to normal tissues of rat brain (135). The study by Meng *et al* provides for the first time, the use of an in vitro methodology that reproduces the preferential accumulation seen in vivo for colloidal materials into a variety of tumour types documented back in the early 1960s (163). This preferential uptake ensures minimal exposure of healthy brain cells to the drug, minimising toxicity (96).

1.5.1 Strategies to deliver drugs locally

In this section example of some of the strategies to deliver drugs locally to the brain is discussed in this section.

1.5.1.1 Intrathecal/intra-cerebrospinal therapy

The CSF is secreted by the choroid plexus of the lateral, third and fourth ventricles and some extra choroidal sources (164). Its main functions include regulation of the outflow of metabolites and catabolites out of the brain, movement of hormones, regulation of extracellular environment and protection for the brain from the movement of the body (165). The CSF occupies the subarachnoid space and fills the ventricles and it is produced and excreted to the blood 4-5 times daily. The flow of CSF does not encourage the flow of nutrients or molecules into the brain rather it is responsible for the drainage of liquid from the brain (166).

Advanced treatment methods have led to better overall survival rates but with relapses observed due to metastases in the CNS, spine and through leptomeningeal dissemination (167). Intra CSF therapy can help to treat metastases especially due to these recurrent tumours particularly those localised to the CNS. Drug delivery is achieved via this route by injection into the ventricles or into the cerebrospinal canal (117). Usual CSF exposure to systemic chemotherapy is low, intrathecal therapy can help to improve this while avoiding toxicity. Fleiscchack *et al* observed over a 100 fold higher CSF peak levels with intraventricular etoposide compared with etoposide administered via intravenous infusion (168). Despite this higher CSF drug concentration negligible systemic or CNS side effects were recorded. The lower systemic toxicity may likely be due to the lower amount of drug administered; 400 mg/m² over 96 hours for systemic compared to 0.5 mg daily for 5 days intraventricularly.

Some problems that may affect effective drug delivery via this route is low and inadequate drug penetration into the brain parenchyma. This is because the clearance of drug from the CSF is faster than its rate of diffusion into the brain parenchyma. Clearance of drug from the CSF is due to high CSF turnover that causes bulk flow of CSF from the CNS thus

leading to insufficient CSF drug levels (169). The function of efflux transporters present on the blood-cerebrospinal fluid barrier and absorption of CSF by arachnoid villi also contribute to drug clearance from the CSF (170).

Furthermore, administration to the CSF is painful and involves inconvenient intra-lumbar injections which is not desirable especially in children. The use of an ommaya reservoir has been used to mitigate against the pain of intra lumbar injection. This ensures continuous and more uniform drug access to the CSF. However, the disadvantage of an invasive procedure to place the device is a problem here too. Liposomal cytarabine (Depocyt®) is the only approved drug for this route of administration. It contains cytarabine formulated inside Depofoam; small particles of a synthetic lipid material (171). It is retained in the CSF due to its large particle size and release is sustained which reduces the need for the repeated lumbar puncture. Due to high manufacturing costs and side effects leading to a need for concomitant use with steroids, Depocyt® has now been withdrawn from the market (148).

1.5.1.1.1 Properties of drugs suitable for intrathecal delivery

Conroy *et al* identified clinical, biological and physicochemical properties that can be used to choose ideal drugs for intrathecal administration, particularly for the bypass of the BBB (7). These properties are summarised in Table 1-5. It is in agreement with these properties that the choice of etoposide, etoposide phosphate and teniposide were made. However, they do not have all the properties stated and one of the aims of the project is to improve the important physicochemical property of having ‘an adequate drug dosage in an ideal delivery system’.

Group	Properties
Clinical	Non-irritant to the brain
	No risk of neurotoxicity
	Activity against specific tumour
Biological	Cell cycle non-specific
	Not susceptible for removal via CSF active transport
Physicochemical	Adequate drug dosage in an ideal delivery system
	delivered to the CSF in active form or requires predictable activation such as hydrolysis
	have properties that reduce the ability to cross the BBB

Table 1-5 Summary of the properties to consider when choosing drugs for intrathecal delivery, taken from (7).

Intrathecal administration has been used to deliver methotrexate, cytosine arabinoside and corticosteroids. For young children with MB, intrathecal methotrexate is used to avoid radiotherapy (164). Some of the other drugs being investigated and some that have gone to phase one trials include Topotecan, Busulfan, Etoposide. Etoposide is one of the drugs to be used in this study, its choice is mainly because it is non-neurotoxic and it is delivered in its active form. The drugs and their properties will be further discussed in this introduction (see Section 1.6).

1.5.1.2 Interstitial, intratumoral and intracavitary delivery

Interstitial, intratumoral and intracavitary are local drug delivery routes that deliver drugs to the extracellular matrix rather than via the vasculature, thereby bypassing the BBB. Interstitial involves placing drugs directly into the interstitial compartments of the tissues surrounding the

tumour. In the case of intra-tumoral administration, the drug is delivered directly to the tumour tissue while intra-cavitary involves drug delivery into the body cavities. Drug delivery into resection cavity post-surgery creates a reservoir for the drug aiding increased, slow and controlled access of the drugs to tumours (74). The primary aim of local delivery is to ensure higher exposure to the target site by greatly reducing systemic drug exposure. Improved drug delivery can be further achieved by incorporating the drug in a delivery system that controls its release in the vicinity of the tumour cells.

This delivery method is established in some brain tumours, for example, Gliadel® is used in Glioblastoma (7,42). Gliadel wafer is a carmustine impregnated disc-shaped biodegradable implants designed to release its payload slowly after being placed in the resection pocket. The drug is contained in polymer microparticles which are compressed into a wafer. The drug is slowly released into surrounding tissue as the wafer degrades to destroy leftover local tumours cells close to the resection pocket (172), this results in a high local drug level without the associated systemic toxicity which occurs when the drug is systemically administered.

The modest 2-3 months improvement in survival have been plagued with challenges. Some of the major drawbacks to the use of Gliadel is the development of complications such as cerebral edema, infection and seizures (173,174). In addition to this, the extent of drug diffusion from the implantation site is limited to about 2 mm and this limits drug efficacy (166). Wang *et al*, reported a low BCNU penetration of 5mm after 100 hours of wafer implantation as the drug gets reabsorbed into the systemic circulation before appreciable diffusion occurs (157).

For post-surgical delivery where the aim is to kill the remaining tumour left behind by surgery, selectivity between the normal cells and tumour cells is an important requirement. It is also important to note that

Carmustine can pass the BBB, so systemic treatment with this drug does have a significant effect. For drugs such as etoposide which do not pass the BBB, the ability to use local delivery to get the drug to the site will enable a wider range of drugs to be used to treat the disease.

Nanoparticles can be useful for these routes of drug delivery provided adequate drug amounts are encapsulated and retention in the nanoparticles is sustained until the nanoparticle reaches the target cell. In addition to this, the loaded drug should be non-neurotoxic, potent against the intended tumour. The chosen carrier should be non-toxic, biodegradable into by-products that are non-toxic and effectively cleared from the body (117).

Though not intended to be used in this project, convection enhanced delivery (CED) is an alternative local delivery strategy employed to bypass the BBB by directly injecting drugs through microcatheters placed directly into the brain (175). This technique applies a pressure gradient which allows tissue perfusion ensuring wider drug distribution (176). The intended nanoparticles on this project with high drug loading and retention could also be an option for this strategy.

1.6 Etoposide, Etoposide phosphate and Teniposide

Podophylotoxins are naturally occurring lignans obtained from the root and rhizome of *podophyllum sp* (177). Their use which dates back many centuries includes anti-tumour and antiviral applications (178). The problem of toxicity has not only affected their use but has prompted further investigation into the synthesis of newer derivatives (179). The need to improve therapeutic efficacy and reduce toxicity led to various alterations of the podophyllotoxin backbone (180).

Etoposide (VP-16, 4'-dimethyl epipodophylloxin-9-[4,6-O-ethylidene-beta-D-glucopyranoside) and Teniposide (VM-26, 4'-demethylepipodophyllotoxin-9-[4,6-O-(R)-2-thenylidene-beta-D-glucopyranoside] act in the late S and early G₂ phase by inhibiting topoisomerase II, the enzyme responsible for coiling and uncoiling of DNA (Figure 1-12) (180). They do this by stabilizing the DNA-enzyme complex hence causing a break in the cell cycle leading to apoptosis. Teniposide is a more potent, more hydrophobic analogue of etoposide (179) while etoposide phosphate is a pro-drug of etoposide which is water soluble (up to 20 mg/ml). It has a similar pharmacokinetic and toxicity profile and it is formulated to overcome the water solubility problems encountered with etoposide (179). The *in vivo* conversion of etoposide phosphate to etoposide by alkaline phosphatases occurs rapidly (177,179).

As a moderately hydrophobic compound widely used in the treatment of many malignancies including paediatric brain tumours, etoposide has a molecular weight of 588, oral bioavailability of 24-75% and elimination half-life of 4-8.8 hours (179). Like many other anticancer agents, etoposide has toxic side effects like nausea, myelosuppression, vomiting and alopecia, due to its effect on normal and off-target tissues, moderate potency and development of drug resistance (177,181,182). Hande stated that topoisomerase II inhibitors have affinity for the multidrug resistant phenotype of glycoprotein P (181).

The effective use of etoposide in the chemotherapy of tumours depends on continuous exposure of tumours to the anticancer agent. The secondary leukaemia adverse effect of etoposide is therapy related and was reported to be more prevalent with higher doses and repeated applications of etoposide (182). Etoposide preparations currently in the market require the use of excipients which are associated with many toxic effects such as hypersensitivity reaction, bronchospasm, hypotension (179,183). Typically, a vial of etoposide contains 100 mg etoposide, 10 mg citric acid, 3.25 mg

polyethylene glycol 300, 500 mg polysorbate 80 and made up to 5 ml with benzyl alcohol (179).

In the case of teniposide, it is a more hydrophobic drug and has been solubilised in existing formulations using cremophor, which has its own drawbacks due to toxicity. Toxic reactions to cremophor include hypersensitivity reactions, neurological toxicity and possible drug interactions (184).

The adverse effects of the excipients used in conventional formulations creates a need to develop new delivery strategies for etoposide. In addition to this, their use in the treatment of malignant brain tumours is limited because of the existence of the blood-brain barrier, as they do not cross the BBB. To further improve the usage of etoposide, etoposide phosphate and teniposide various novel drug delivery strategies have been used like nanosuspensions (185) and polymer micelles (186). These involve etoposide incorporation in polymeric nanoparticles, solid lipid nanoparticles, cationic liposomes and liposomes (76). These novel strategies not only improve the Pharmacokinetic properties of the drugs but also increase therapeutic efficacy (187). They can be used for passive targeting via the EPR effect (76) or active targeting in which specific ligands are bound to the drug (188). Nanoparticle formulations can improve bio-distribution and circulation times of etoposide (189).

In recent years there has been a spate of interest in the incorporation of etoposide and teniposide into various novel drug delivery systems. An important component of the NPDS is the polymer. In the preparation of etoposide/teniposide incorporated nanoparticles, PLGA is the most commonly used (189–191). Others include poly(ethylene glycol) and sebacic acid (PEG-PSA) (192), poly(butylcyanoacrylate) and PLGA-PEG (193). The use of PLGA has been associated with biphasic release (194)

which is characterised by an initial burst release. Hence the initial burst release reported by (190,195) was not uncharacteristic.

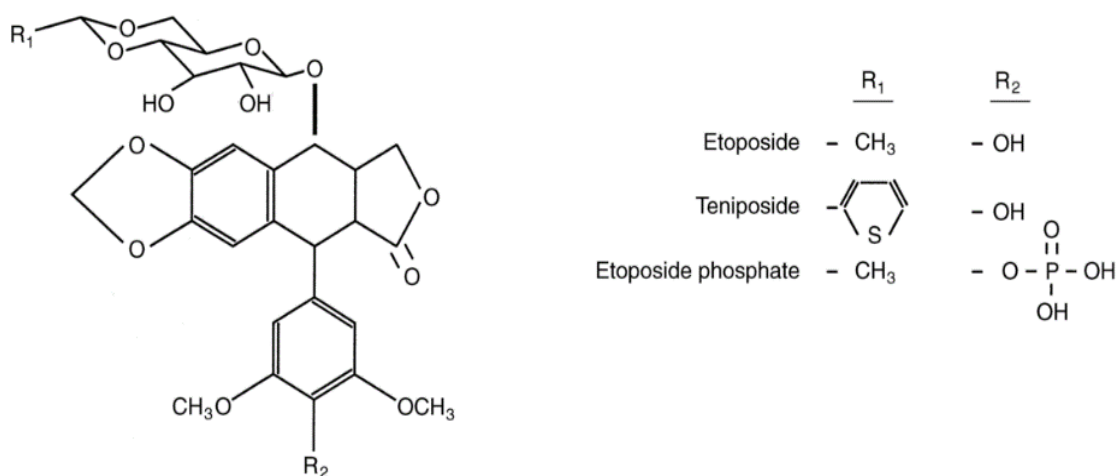


Figure 1-12 The chemical structure of etoposide, etoposide phosphate and teniposide (179).

1.7 Rationale for the project

1.7.1 The drugs and polymer

Etoposide is a commonly used anticancer agent, that has been identified to be non-neurotoxic hence ideal for local brain delivery (168) and has demonstrated efficacy against MB in patients (196,197). After systemic or oral administration, less than 3% of etoposide reaches the CSF, this shows a need to explore the use of local delivery methods for the delivery of etoposide. Fleischhack *et al* assessed the feasibility of intraventricular delivery of etoposide to patients with metastatic brain tumour and found it safe to use with minimal toxicity (168).

Etoposide phosphate is a prodrug of etoposide that requires hydrolytic conversion to etoposide, in the cells. Etoposide phosphate is rapidly converted by phosphatases. Teniposide is a drug in the same class as etoposide, but more hydrophobic molecule and has been shown to be more potent than etoposide (117). The three drugs provide a variation in hydrophobicity, water solubility and potency, hence, their interactions with the different PGA polymers used in the study and can then be compared.

Poly(glycerol adipate), the polymer of choice has a pendant hydroxyl group which can be substituted to give polymer variants with different physicochemical properties (94). Previous work demonstrates preferential uptake into DAOY spheroids and organotypic brain slices (198) of PGA nanoparticles and reported low drug loading into these substituted PGA polymers (89,117,199).

1.7.2 The delivery system

The development of a delivery system for a disease should be guided the specific conditions of the disease, this is because the delivery system will be developed to capitalise on the physiological changes that occur due to the disease. The ideal delivery system would be made of biodegradable materials, have the right physicochemical properties, containing the right drug in a sufficiently effective quantity and with the ability to deliver the right quantity of its payload to the right site at the right time.

As all MB patients are treated by the surgical removal of tumours, this provides an opportunity for drug administration route, with an appropriately designed delivery system. This project explores this opportunity to develop an adjuvant therapy that treats the residual tumour tissue, left behind by the surgery. Figure 1-13 shows how nanoparticles could be used in a local delivery to the tumour pocket.

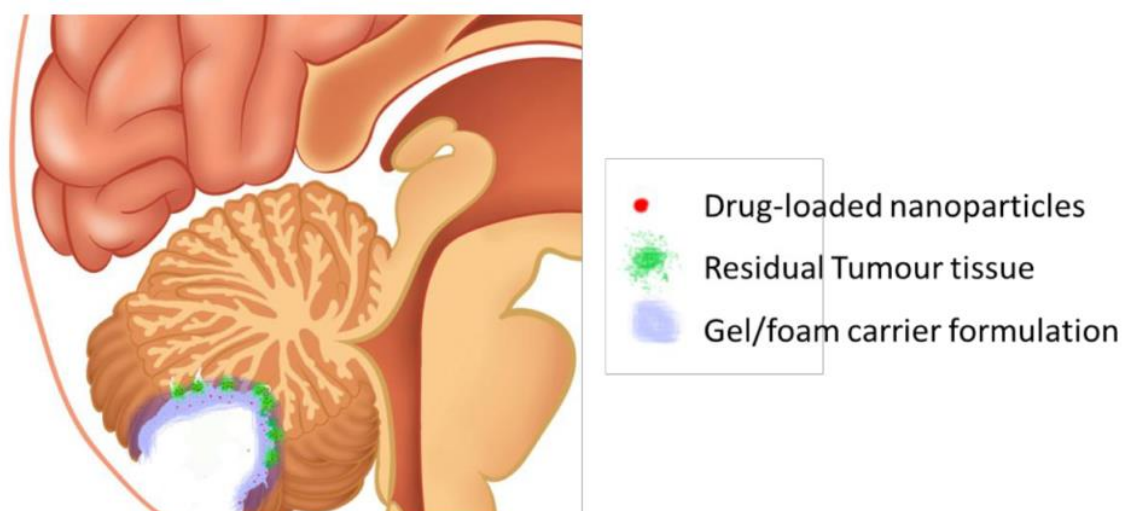


Figure 1-13 illustration of the proposed method of local delivery of the nanoparticles. The delivery is into the pocket created after surgical tumour resection. The drug loaded nanoparticle (red) can be delivered using a gel or foam-based carrier (grey) onto the residual tumour (green). This is important because residual tumour is usually the cause of recurrence, the carrier is important to hold the NPDS in place in order to avoid being washed away before uptake by the tumour cells. The preferential uptake of the PGA NPDS by MB cells is exploited in this project taken from Ivanov (2014) (117) modified from Janet Fong 2009.

The gel or foam-based carrier administered into the resection cavity holds the formulation close to the surface of the brain. The cut surface of the brain allows direct access of the nanoparticles to the brain tissue rather than the vascular system. This access is to the residual tumour cells, the brain cells and the extracellular matrix, so the nanoparticles can diffuse into this tissue mass and take advantage of the enhanced endocytosis demonstrated by tumour cells compared to normal brain cells in 3-D culture conditions.

Firstly, in chemotherapy, the systemic route to the tumour which relies on the EPR effect is the most commonly used route to passively deliver drugs to tumours and it has not been very successful (154). The use of local delivery though desirable still requires further modification to ensure efficient delivery. To improve the outcome with post-surgical residual cancer cells local delivery with improved selectivity is desirable. This

project exploits the results from Meng *et al*, which demonstrated the preferential uptake of PGA NP by MB cells. She demonstrated that RBITC labelled PGA nanoparticles were taken up six times more by tumour spheroids compared to normal tissues of rat brain (135). This preferential uptake ensures minimal exposure of healthy brain cells to the drug, minimising toxicity (96).

This proposed delivery system is made up of nanoparticles expected to entrap such chemotherapeutic agents and retain them long enough for preferential uptake by MB cells. Delivering the drug loaded NPDS into the post-surgical tumour pocket serves to prevent tumour recurrence that is caused by residual tumour. The NPDS must retain the drug long enough to reach the tumour cells before uptake into these cells. The release of drug from the nanoparticles on this project is expected to occur via polymer degradation by the action of enzymes. The expected steps involved in the process are:

1. Intact nanoparticles are preferentially taken up into the tumour cells.
2. Polymer degradation occurs in the lysosomes by naturally occurring enzymes to release the drug.
3. The released drug diffuses out of the lysosome to its pharmacological target site to effect its usual drug action.

The delivery systems should be prepared to suit specific drugs as there is no one size fits all delivery system for all drugs. Since drug delivery strategies are expected to be formulated on disease by disease basis, the design of Nanoparticles should be based on the specific characteristics and behaviour of the tumour. However, a successful nanoparticle design can then be adapted to fit other disease conditions and delivery methods.

1.7.3 The Required Nanoparticle specification

In the absence of any interactions between drug and polymer, the release of drugs from nanoparticles is expected to occur in minutes. So, on this project, based on the emphasis on drug-polymer interactions, the 'definition' of burst release is the time it takes for the drug to diffuse out of the nanoparticle if it is not held in by any interactions. It contributes to low delivery efficiency of nanoparticles as it may limit the amount of drug getting to target site. One of the goals on this project is to minimise burst release.

In addition to this, the exploitation of some properties of NPs to improve delivery by taking a local route rather than systemic route is the proposed strategy on this project. The required nanoparticles will:

- Be administered locally post-surgery.
- Have a size small enough and have a coating that allows tumour penetration and extravasation.
- Incorporation of a sufficient amount of drug.
- Retention of the drug long enough for it to reach its target tumour cells (ideally greater than 24h).
- Be preferentially taken up by tumour cells rather than normal brain cells.
- Release drug within the tumour cells after polymer degradation by naturally occurring enzymes in the lysosomes.

The preparation of nanoparticles has not been based on established guidelines and methodologies (200), especially in the choice of polymers for a drug. The usual process of making nanoparticles is to prepare them first and then characterise them to see encapsulation and the release rate.

Also, the exploration and determination of contributory factors to the drug loading and release results are determined after preparation and characterization.

In Conroy's review, one of the properties of an ideal drug for local delivery includes presenting the drug in the right delivery system (7). This project was set up to tackle the challenges involved in getting the 'right' delivery system. A system that would work as the proposed delivery system having the right amount of drug and retained for an adequate length of time. Low drug loading is a common occurrence with NPs and fast release within minutes is the usual expectation for NPs. This is because compared to microparticles nanoparticles have a smaller diffusional distance for the drug to pass through the particle matrix and out into the surrounding medium (98).

This project is based on the hypothesis that interaction between drug and polymer can aid drug retention and slow down release. It was set up as a reverse engineering process of the steps involved in the preparation of polymeric nanoparticles. The focus is the informed choice of polymers for specific drugs and is based on the hypothesis that interaction between drugs and polymer have an impact on drug incorporation and drug retention by the polymers. The aim is to tackle the physicochemical aspects of improving the drug loading and release of the nanoparticles.

One of the endpoints is the understanding of the interaction between a drug and polymer and the effects of the interactions on the loading and release and to use this knowledge to develop an optimum and suitable NPDS with the right parameters and properties for local delivery as adjuvant therapy in the treatment of MB.

The composition of the nanoparticles is important to the success of the nanoparticle in drug delivery (201) and this project mainly focusses on

getting the right drug-polymer composition that ensures the right amount of drug gets delivered and the retention is long enough for the nanoparticles to reach the cancer cells and for internalisation into the cells to occur.

1.8 Aims

This work aims to understand the behaviour of the PGA class of polymers (Figure 2-1) with these three drugs; etoposide, etoposide phosphate and teniposide. The goal is to assess the ability of the polymer to delay the release of the drug and extent of drug retention by the polymer. This was done by developing a method to assess the innate affinity of drugs for the polymers and to assess if this translates to better nanoparticles with improved release and loading.

1. To screen known anticancer agents (Etoposide, Etoposide phosphate and Teniposide) against a set of substituted polyglycerol adipate polymers. This will lead to an improved understanding of the interactions between various substituted polyglycerol adipate polymers and the anticancer agents.
2. To choose the best polymer after screening for the preparation of an optimum NPDS with higher drug loading for these anticancer agents.
3. To prepare and characterize the nanoparticles prepared from the drug-polymer matches.
4. To carry out cytotoxicity experiments to compare the cytotoxicity of the free drugs with the nanoparticle formulations.

1.9 Overview of the Project

This project is a preclinical research that is a part of the translational journey to improving the delivery of widely used drugs. I explored ways to improve some key properties of nanoparticles. However, there are further key stages to explore before the formulations here get to the clinic.

Determination of polymer-drug combinations was done by making a dispersion of drugs in insoluble polymers then carrying out release experiments from thin films to determine the drugs with the best retention of drug. The polymers have different functionalities with the ability for new chemical interaction.

The use of films made in form of solid dispersions was informed firstly, by the need to have a quick method to conduct the release experiments instead of fully preparing and then characterising nanoparticles which can be very time consuming. Secondly, the incorporation of drugs into films can be controlled and the specific amount of drug present in the film is known.

A small number of techniques were then used to look in more detail at possible interactions between drugs and polymers. Firstly, FTIR was used to monitor the changes to the peaks and the peaks positions in the drug and polymers and subsequently in the drug-polymer films after interaction. These changes when analysed helped to pinpoint some interactions involved.

Secondly, the measurement of the contact angles of the drugs, polymers and drug-polymer films and then monitoring the changes to the contact angle as observed in the films. Any changes in contact angle on the preparation of drug-polymer films could be used to infer changes in the association between polymers and drugs.

The polymer drug combination made from the observed interactions were then made into nanoparticles using the interfacial deposition method, to determine if this resulted in nanoparticles with improved drug loading and drug retention compared to previously reported formulations.

Finally, a novel type of nanoparticle was prepared, polymer coated drug nanoparticles to further enhance the drug loading potential of these formulations. Cytotoxicity experiments were conducted on both types of nanoparticles to determine how these different types of nanoparticles may behave as a drug delivery system in terms of potential therapeutic activity.

Chapter 2

2 Experimental materials and methods

2.1 Materials

2.1.1 Drug-polymer screening and nanoparticle preparation and characterization

Etoposide (CAS 33419-42-0) was obtained from Cayman chemical company, teniposide (CAS 29767-20-2) was obtained from Sequoia research products, UK, etoposide phosphate (Bristol Meyers Squibb Company, USA), HPLC grade acetone, acetonitrile, dichloromethane (DCM) and methanol were obtained from Fischer Scientific UK, dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK), sodium cholate (Alfa Aesar, UK), phosphate buffered saline (PBS, pH 7.0) was obtained from AppliChem PanReac company ltd.

Polymers: Poly(glycerol adipate) (PGA), PGA-poly(ethylene glycol) (PGA-PEG), 40% C8 substituted (PGA40%C8), 40% C18 substituted (PGA40%C18), 50% Phenylalanine substituted (PGA50%Phe), 50% Succinic anhydride substituted (PGA50%SA), 85% C18 substituted (PGA85%C18), 30% Cholesterol substituted (PGA30%CH) and 50% Tryptophan substituted (PGA50%TRY) were prepared by Vincenzo Taresco at the School of Pharmacy University of Nottingham. The polymers were fully characterised and information such as their molecular weight, glass transition temperature, NMR and IR data can be found in Taresco *et al* (94). The calculated log P values of the repetitive units of the polymers are listed in Table 2-1 and the structures of the polymers with their substituted functionalities are shown in Figure 2-1.

Polymer	LogP of Repetitive units
PGA	0.96
PGA ₅₀ %SA	-0.03
PGA-PEG	0.71
PGA ₅₀ %Phe	1.06
PGA ₅₀ %TRY	1.12
PGA ₄₀ %C8	2.27
PGA ₄₀ %C18	5.45
PGA ₃₀ %CH	6.06
PGA ₈₅ %C18	6.06
Etoposide phosphate	0.51
Etoposide	1.16
Teniposide	2.78

Table 2-1 The log P values of the repetitive units of the polymers were estimated using Chem draw software. The log P values of the drugs were obtained from (202–204). The log P is a parameter that reflects the hydrophobicity of a compound.

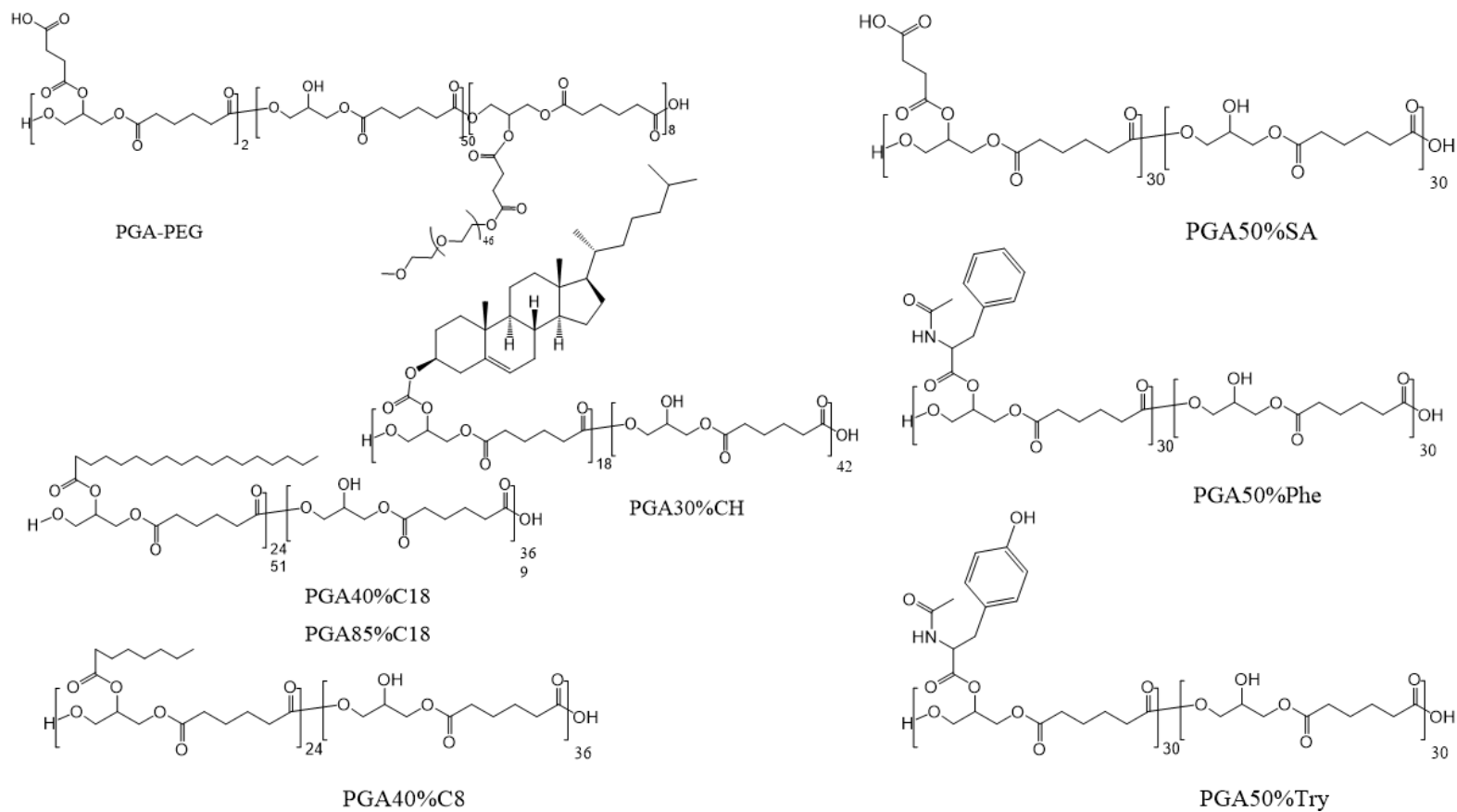


Figure 2-1 The structure of substituted polymers showing the different substituted functionalities, the larger the alkyl group in the substituent the more hydrophobic the polymer. The polymers have new physicochemical properties and are able to interact with the drugs in different ways and to varying extents.

2.1.2 Cell line and culturing

UW228-3 medulloblastoma cell line (205) was obtained as a gift from Prof. J.R. Silber (University of Washington, Seattle, USA) through the Children's Brain Tumour Research Centre at the University of Nottingham. The UW228-3 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (DMEM/F12) medium supplemented with L-Glutamine (2 mM), sodium pyruvate (100 mM) and Fetal Bovine Serum (FBS) (15%). The flasks were incubated at 37°C in an atmosphere of 5% CO₂. The cells were received at passage 22, but most experiments were carried out at between passage 25-30. The doubling time of UW228-3 cells has been reported to be 29 ±2 hours and the cells were reported to grow exponentially for at least 48 hours in 15% FBS supplemented DMEM medium (205). However, on this project, I observed the cells reached 80% confluence at about 48 hours.

2.1.3 Cytotoxicity Assay

All cell culture reagents such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, DMEM/F12, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), triton X-100, trypan blue, trypsin ethylenediaminetetraacetic acid (Trypsin-EDTA) were all obtained from Sigma Aldrich, UK.

2.2 Methods

2.2.1 Drug-polymer screening

2.2.1.1 Drug release from drug-polymer films

The first set of experiments on this project were carried out to assess the interactions between the drugs- etoposide, etoposide phosphate and teniposide and polymers. Drug-polymer films were prepared from all three drugs and the nine polymers. This was an attempt to have drug and polymer interact based on their intrinsic affinity for one another. This affinity was expected to result from the physical and chemical interactions between the different drugs and polymers. Firstly, the extent of drug retention by each of the polymers (PGA backbone, PGA-PEG, PGA85%C18, PGA30%CH, PGA40%C8, PGA40%C18, PGA50%TRY, PGA50%Phe, PGA50%SA) was determined by examining the amount of drug released after 24 hours. It is expected that the diffusion of drugs through thin films of polymer would be very rapid. Therefore, if drug release takes a longer time, it implies some sort of interaction between drug and polymers, and the slower the rate of release, the greater the presumed strength of interactions between drug and polymer.

2.2.1.1.1 Release experiment

Drug-polymer films (40%^{w/w}) were prepared by dissolving drug and polymer in a common volatile solvent followed by solvent evaporation (206). 40%^{w/w} was used because drug percentages lower than 40% were not detectable in the dissolution media. Etoposide, teniposide and etoposide phosphate (5.3 mg) were separately dissolved in acetone (10 ml). Polymers (5.3 mg) were also separately dissolved in acetone (10 ml). Polymer solution (0.6 ml) was added to drug solution (0.4 ml) in a vial. A

drug solution containing 100% drug (1 ml) served as control. The mixtures were placed overnight in the fume hood, where the solvent was allowed to evaporate leaving behind a drug-polymer film present as thin coating at the bottom of the vial.

Sodium cholate (1%^{w/v}) in Phosphate buffered saline (PBS) (10 ml) was added to etoposide and teniposide containing films, while PBS (5 ml) was added to etoposide phosphate containing films. These release media were chosen for the drugs to maintain sink conditions. Aliquots of the release medium (0.5 ml) were withdrawn at regular intervals (1, 2, 3, 4, 5 and 24 hours) and replaced with an equal volume of fresh medium. The samples were stored in the freezer at -20°C and analysed using High performance liquid chromatography (HPLC) (Agilent 1050 series) for teniposide and etoposide and Ultraviolet Spectroscopy (Varian Cary 50 Bio UV-VIS Spectrophotometer) at 281nm for etoposide phosphate. The remaining volume of the sample was also kept refrigerated and later used to calculate the percentage (%) retained.

2.2.1.1.1 Solubility of Etoposide and Teniposide in 1% Sodium cholate PBS

Sink conditions were maintained in accordance with the specifications in the British Pharmacopoeia (BP) (116). To achieve this sink conditions and in cognizance of the low water solubility of etoposide and teniposide, there was a need for an appropriate dissolution media. Ivanov (2014) demonstrated the suitability of 1% sodium cholate in PBS for the determination of drug release of teniposide with fluorescence and HPLC (117). On this project, the kinetic solubility of both teniposide and etoposide was determined. This was done by placing excess amount of etoposide(1 mg) and teniposide (1 mg) in separate vials containing 1% sodium cholate PBS (1 ml) and placed in a water bath at 25°C for 24 hours with constant shaking. The solubility was found to be 181 µg/ml and 153

µg/ml for etoposide and teniposide respectively. Each film contained 212 µg of drug.

2.2.1.1.2 High performance liquid chromatography (HPLC)

Etoposide and Teniposide release samples were analysed using an HPLC method previously used by Ivanov (2014) (117). The method involved the use of a C₁₈ reverse phase column Licrospher (250-4 mm) column at 40°C. It was eluted with phosphate buffer (50mM) pH 7.04: acetonitrile 55:45 for teniposide and phosphate buffer (50mM) pH 7.04: methanol 55:45 for etoposide. The flow rate of 1 ml/min was maintained and the column eluent was monitored continuously at 240 nm for teniposide and 281 nm for etoposide. The compounds were quantified by measuring the area of peak in relation to those of standards chromatographed under the same conditions. Teniposide was eluted with a retention time of 3.5 min and etoposide at a retention time of 4.7 min. The limits of detection and quantitation were determined as 3 and 10 times signal/noise ratio was determined using 2.0 to 2.2 min and 3.0 to 3.2 min noise range. Precision was assessed by determining the inter and intra-assay variation and acceptance criteria for the coefficient of variation was CV less than 15% (as stated in ICH guideline (207)), although in most cases the CV% obtained was below 10%. The accuracy was assessed by the resolution of peaks compared with the other substances present in the release media. For robustness, the recovery of the drugs after freezing and thawing was used

2.2.1.1.3 HPLC method validation results

The HPLC method used was validated as in section 2.2.1.1.2 and its linearity, accuracy, precision and robustness were determined. The method was linear, the R² value was 1 and the LOD and LOQ were 0.05 µm and 0.1 µm respectively for etoposide as shown in Figure 2-2. For

teniposide, the method was linear, the R^2 value was 0.9996 and the LOD and LOQ were 0.01 μM and 0.03 μM as shown in Figure 2-3.

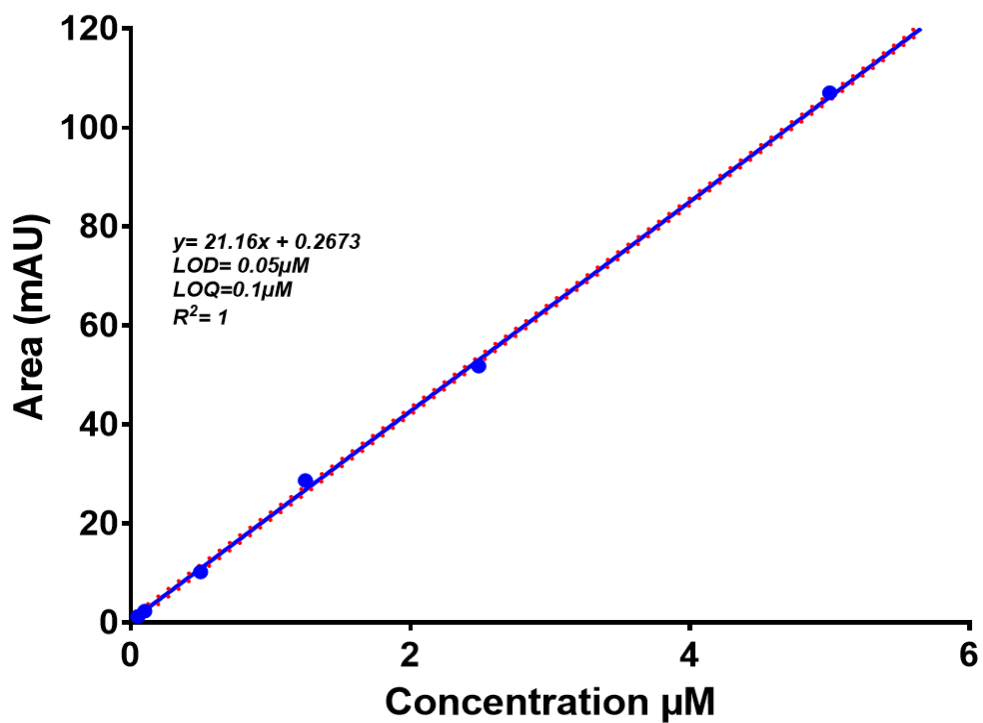


Figure 2-2 Standard HPLC curve of Etoposide in 1% Sodium cholate in PBS

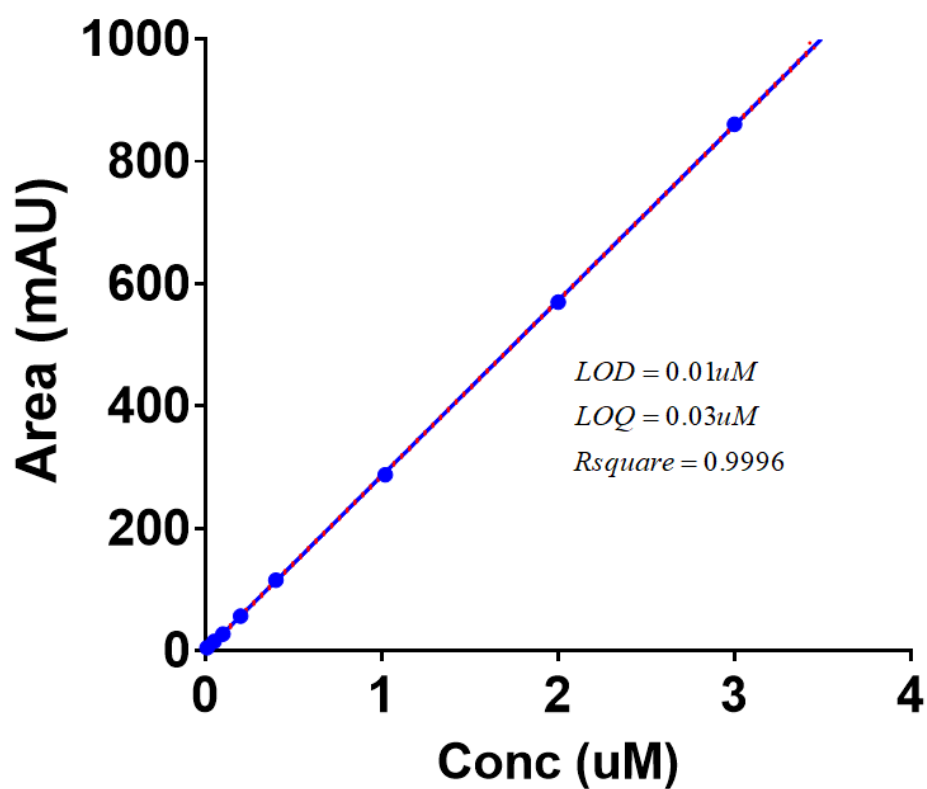


Figure 2-3 Standard HPLC curve of Teniposide in 1% Sodium cholate in PBS

The peak of the released drug was well distinguishable from the other components of the dissolution media. Representative chromatograms of teniposide can be seen in Figure 2-4 below.

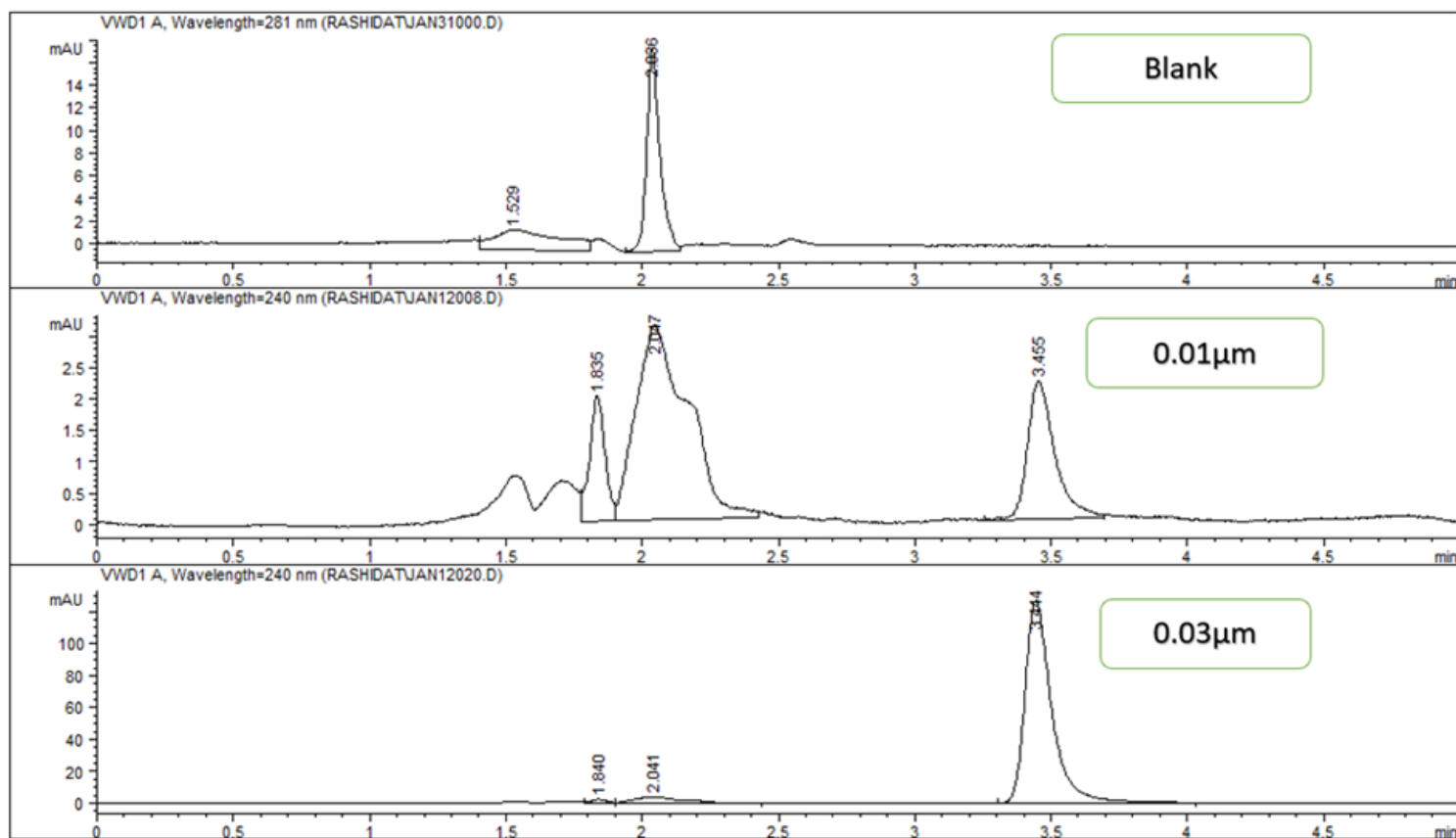


Figure 2-4 Representative chromatograms of blank solution (1% sodium cholate in PBS) and teniposide standards. The retention time was 3.5 minutes and It appeared as a well resolved peak from the blank which had a main peak at 1.5-2.5 minutes.

The intra-assay variation obtained was below 10% for 6 drug levels. The inter-assay precision was also assessed and the coefficient of variation obtained for 6 different separate sets of standards at six different concentrations on three different days are as shown in Table 2-2.

Teniposide			Etoposide		
Concentration μm	Area	CV%	Concentration μm	Area	CV%
0.035	8	10	0.1	2.3	3
0.07	15	14	1	24	3
0.14	29	8	2	50	5
1.4	285	10	5	100.5	7
14	2246	10	10	207	8
28	4476	8	50	1037.5	7

Table 2-2 Inter-assay precision as determined for etoposide and teniposide for six concentrations measured in six separate independent experiments.

The robustness of the method which reflects the stability of the sample of etoposide and teniposide after freezing and thawing is shown in Figure 2-5 and Figure 2-6.

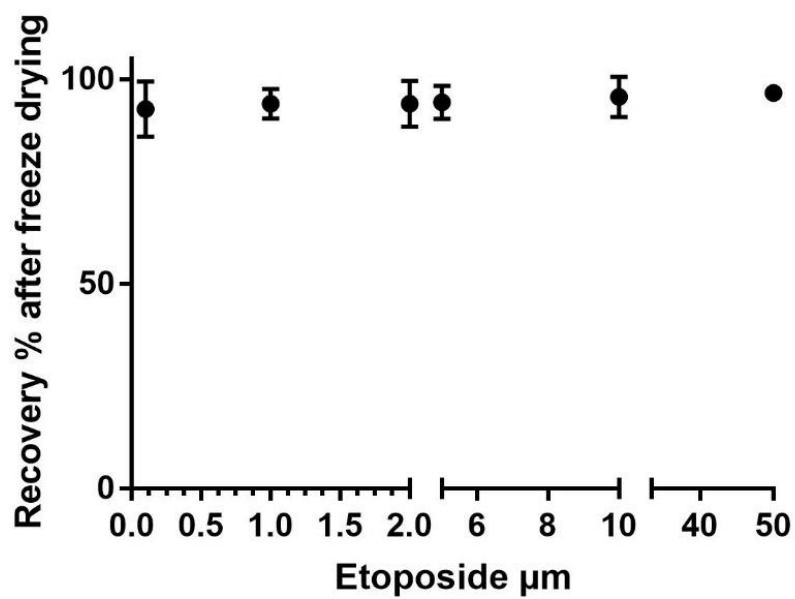


Figure 2-5 Robustness of etoposide HPLC assay; stability of etoposide after freezing and thawing

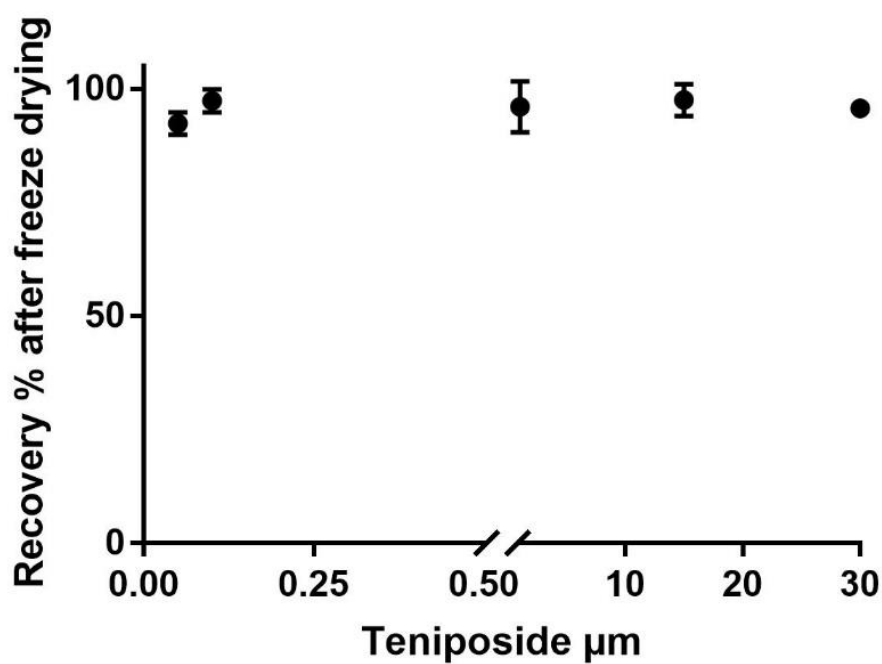


Figure 2-6 Robustness of Teniposide HPLC assay; stability of etoposide after freezing and thawing.

2.2.1.1.4 Validation of Ultraviolet spectroscopy

Etoposide phosphate was analysed using UV spectroscopy. A linear calibration curve was obtained as seen in Figure 2-7. Representative absorbance peaks for increasing concentrations of etoposide is shown in Figure 2-8.

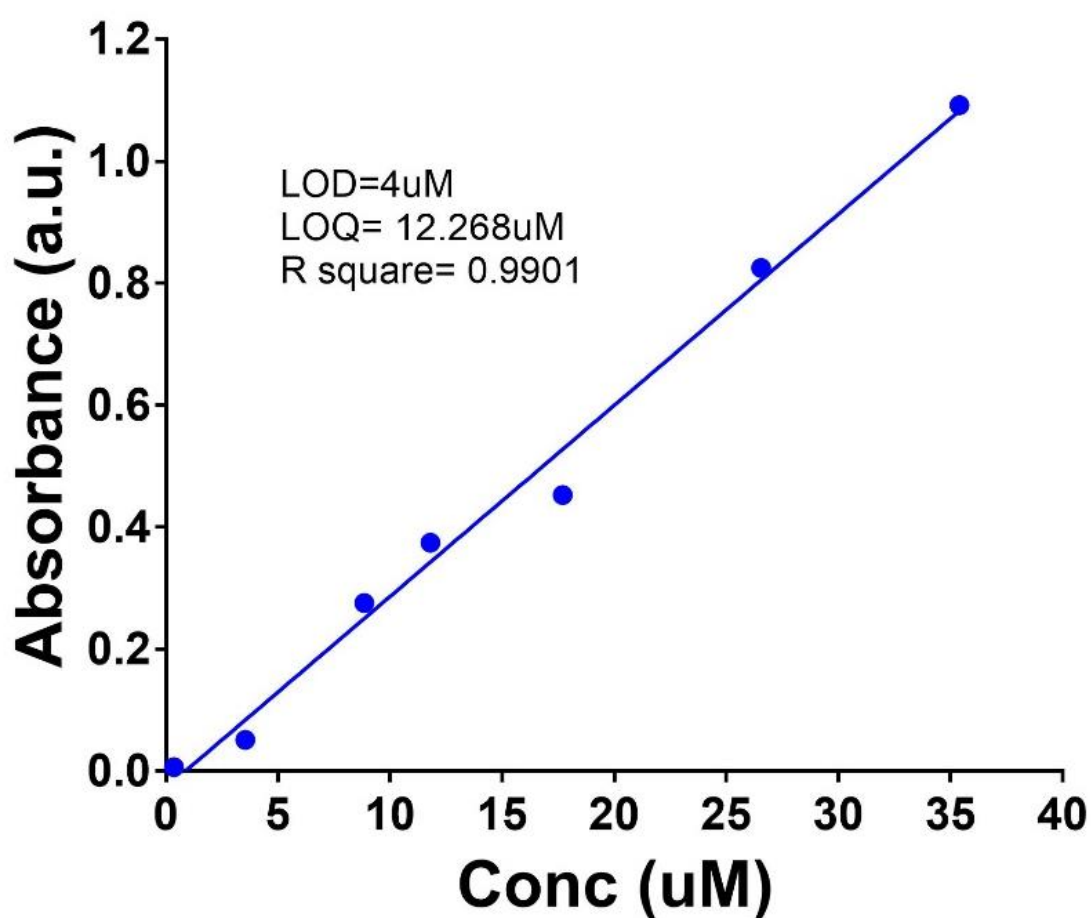


Figure 2-7 Standard UV curve of Etoposide phosphate in PBS at 290 nm

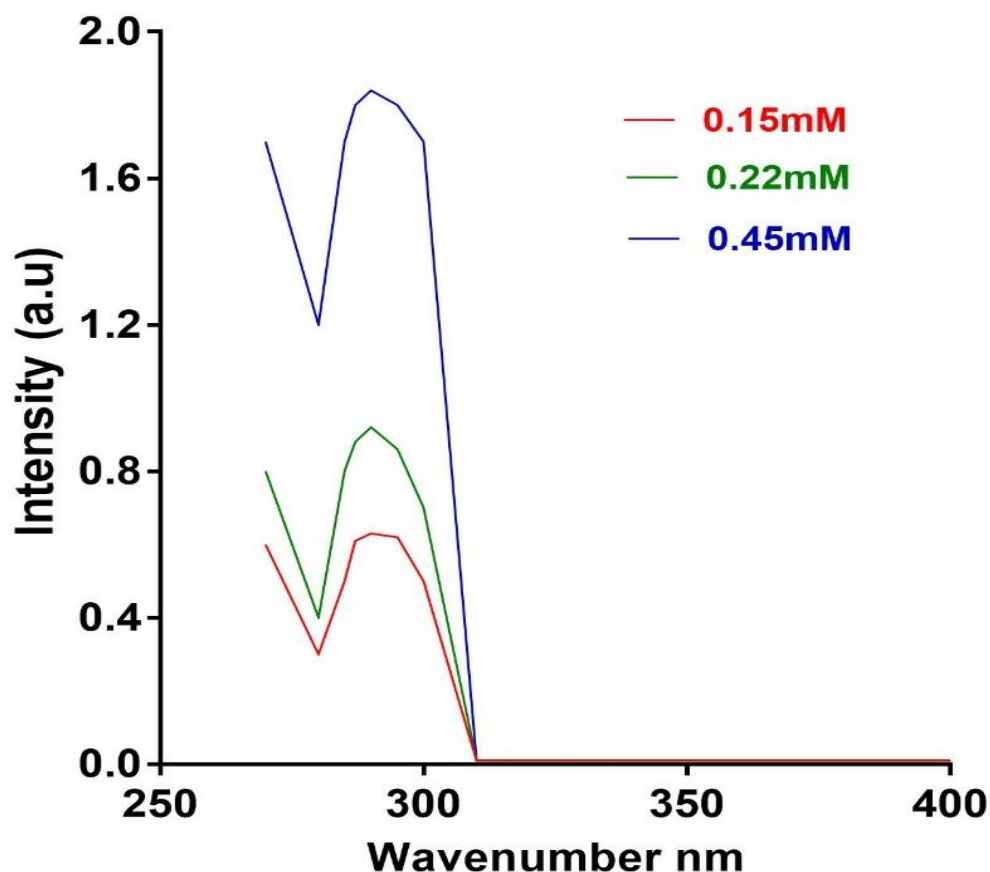


Figure 2-8 Representative UV absorbance peaks for increasing concentrations of etoposide phosphate.

2.2.1.2 Attenuated Total Reflection Fourier transform infrared spectroscopy (ATR-FTIR)

Drug-polymer solutions (40%^{w/v}) in acetone from 2.2.1.1. above were placed on glass slides and the acetone was allowed to evaporate. This was also repeated for solutions containing free drug and polymer. The dried films were scraped, and their FT-IR spectra were recorded with an Attenuated Total Reflection spectrophotometer (Agilent Technologies Cary 630 FTIR) equipped with a diamond single reflection ATR unit. Spectra were acquired with a resolution of 4 cm⁻¹, in the range 4000-650 cm⁻¹ by recording 32 interferograms. Spectra were obtained for drug,

polymer and drug-polymer film and changes in wavenumber were calculated from each spectrum.

Principle:

ATR-FTIR spectroscopy, which involves a beam of infrared light being passed through a crystal that allows total internal reflection Figure 2-9. The IR beam is directed onto a crystal that is transparent, has a relatively higher refractive index and a polished surface. An evanescent wave is generated as the IR beam is reflected from the crystal's internal surface. The wave projects orthogonally into the sample in direct contact with the ATR crystal. Some of the energy is reflected and some absorbed by the sample. The reflected radiation is returned to the detector. The ATR technique has become the standard FTIR sampling technique because it allows faster sampling, high reproducibility and excellent data quality.

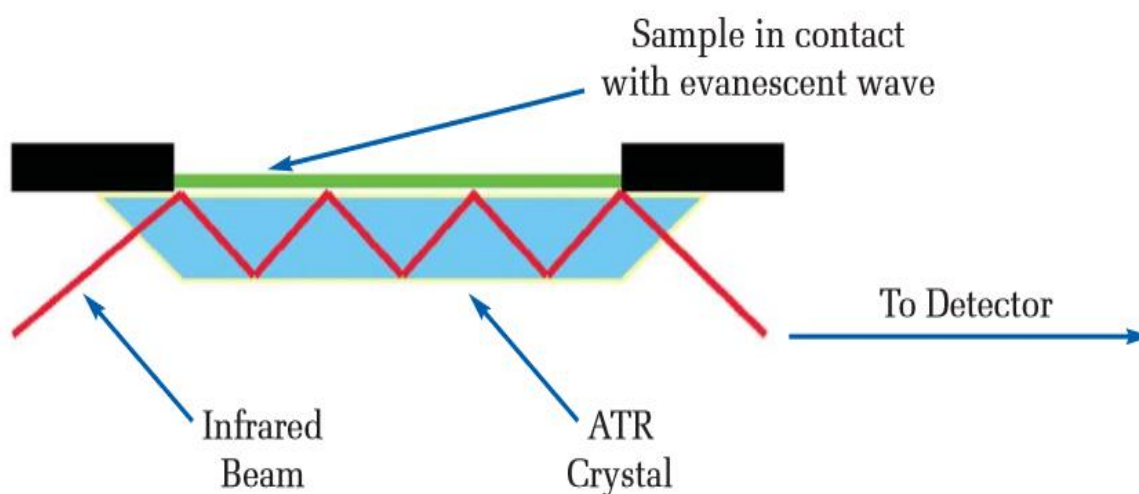


Figure 2-9 Schematic of an ATR-FTIR system. The sample must be in contact with the crystal, adapted from (208).

2.2.1.3 Water Contact angle (CA)

Static contact angle is a measure of the wettability of substances which is a reflection of the hydrophobicity at the surfaces of the substances. Wetting is the first step in the dissolution process and it is influenced by the intermolecular interactions between drugs and polymer. This property reflects the hydrophobicity of the drug and polymer at the surface of the materials.

Contact angle measurements were made using a KSV Cam 200 (KSV Instruments Ltd., Helsinki, Finland) equipped with dedicated software (CAM200). The measurements were carried out at 25 °C. Glass microscope slides were coated with thin drug-polymer films as in section 2.2.1.3 above. The actual measurement was carried out by mounting the slide horizontally on the instrument stand. Using a flattened tip needle attached to a syringe, a small amount (drop) of distilled water was dispensed onto the polymer film. A recording of the tangent line was made, and shape and contact angle were analysed from this. Three measurements were made for each sample.

2.2.2 Nanoparticle preparation and characterization

2.2.2.1 Nanoprecipitation

Nanoparticles were prepared using interfacial polymer deposition following the solvent displacement method (102).

The polymer (20 mg) was dissolved in acetone (2 ml) and mixed with aqueous solution (4 ml) containing etoposide phosphate (2 mg) using a chamber mixer (Pharmacia, 50-60 Hz, 5 MPa). The two phases (organic and aqueous) are delivered through two separate pumps to the chamber mixer from which the nanoparticles formed are collected. The chamber mixer is a device used to ensure uniformity of results. The flow speed used for the aqueous phase is (2 ml/min) double that of the organic phase (1 ml/min) to ensure the correct ratio of components is mixed. The resulting mixture is collected in a vial.

(Etoposide or Teniposide) (2 mg) and polymer (10 mg) were dissolved in acetone (2 ml) and were mixed with water (4 ml) also using a chamber mixer (Pharmacia, 50-60 Hz, 5 MPa) and collected in vials wrapped in aluminium foil.

The vials containing the resulting mixtures were left under magnetic stirring in a fume hood overnight to remove residual acetone. The resulting nanoparticle suspensions were filtered using a 0.45 µm syringe filter before removal of the unincorporated drug as detailed in section 2.2.2.2.

The resulting nanoparticle dispersions were used freshly for cell culture and characterisation or lyophilised for drug loading determination.

2.2.2.2 Removal of unincorporated drug from nanoparticles

The drug loaded nanoparticles were separated from the unincorporated drug by desalting using low pressure gel permeation chromatography using a Sepharose CL-4B column (2.5 x 25 cm, GE Healthcare). A peristaltic pump was used to deliver the samples and eluent (water) which passes through a UV detector (280 nm) and an autosampler that collected 2 ml fractions. The system works on the principle that larger nanoparticles would be unable to enter the cross-linked gel and would be eluted first. The drug which is of lower molecular weight enters the gel pores and consequently has a longer retention time. The separation was verified by a UV detector and chart recorder which showed Nanoparticle and drug peaks with a complete baseline separation on the chart recorder (See Figure 2-10).

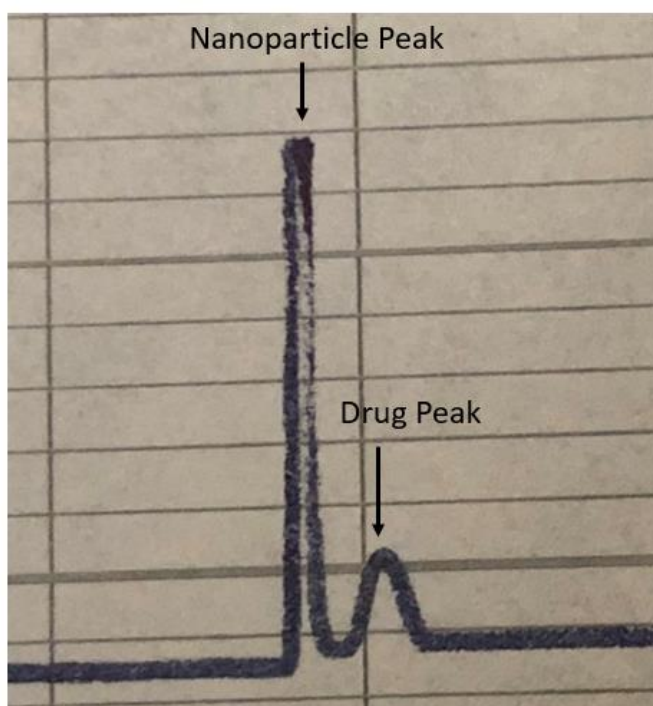


Figure 2-10 Sample peaks obtained on the chart recorder after removal of unincorporated drug using gel permeation chromatography. The first peak is the larger nanoparticle peak while the second peak represents the drug peaks which have a smaller particle size and longer elution time.

2.2.2.3 Determination of Particle size and Zeta-potential

The hydrodynamic diameter was measured using a Malvern Zetasizer NanoZS. The instrument works on the principle of Dynamic light scattering (DLS). The measurements were conducted at 25°C, at a scattering angle of 173°C and laser of 633 nm. The samples were measured in triplicate.

For the zeta potential, the same instrument as above was used; the measurements were conducted at 25°C and three measurements were performed on each sample. Both particle size and zeta potential were measured in HEPES buffer (1 mM, pH 7.4).

2.2.2.4 Drug loading of nanoparticles

The amount of drug loaded into nanoparticles was determined by freeze-drying nanoparticle suspension (1 ml) in a pre-weighed amber HPLC vial. The dried nanoparticles were dissolved in a mixture of DCM: DMSO (1 ml, 50:50) for etoposide and teniposide. Fluorescence spectrophotometry (Varian Cary Eclipse, Agilent) at excitation ($\lambda=284$ nm), at emission ($\lambda=325$ nm) and with slit of (5 nm) was measured against standards which were freshly made.

For etoposide phosphate, the weighed freeze-dried nanoparticles were dissolved in a 1:2 mixture of acetone and PBS. The resulting mixture was then centrifuged at 13500 rpm for 3 minutes. The supernatant was analysed using UV spectroscopy at 290 nm.

For quality control, spiked samples of nanoparticles were subjected to similar treatment and used to determine the suitability of the method for the drug loading determination. The spiking was done with known

amounts of drugs (etoposide, etoposide phosphate and teniposide) and the percentage recovery was determined.

Figure 2-11 shows the Representative peaks of etoposide and teniposide for three different concentrations levels. Standard solutions of known concentrations of etoposide and teniposide in 50:50 DCM: DMSO were analysed using Fluorescence spectrophotometry with excitation ($\lambda=284$ nm), emission ($\lambda=325$ nm) and slit (5 nm) and calibration curves were prepared (Figure 2-12 and Figure 2-13).

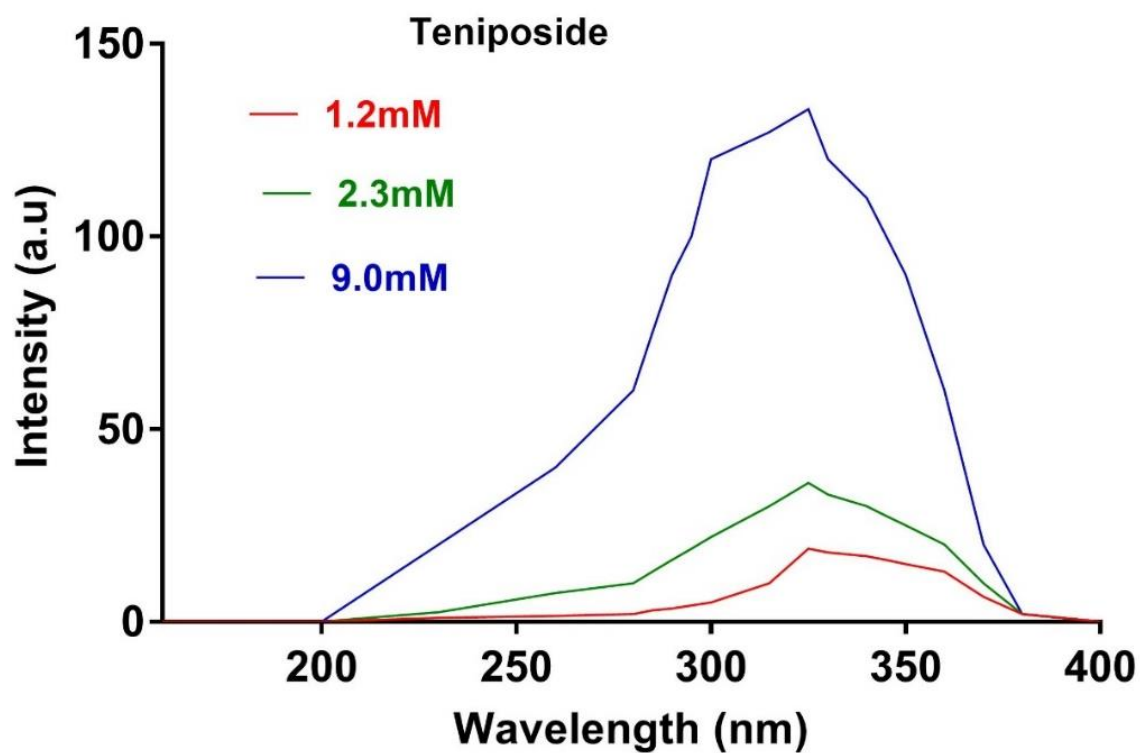
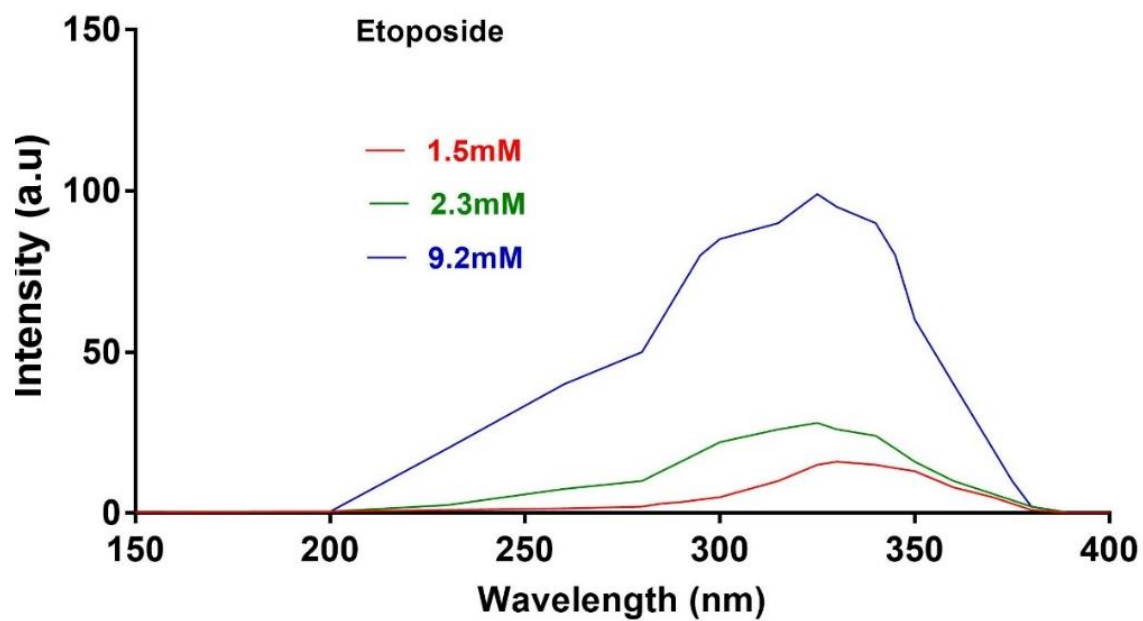


Figure 2-11 Representative fluorescence emission peaks at 325 nm for etoposide at 1.5, 2.3 and 9.2 mM for etoposide and 1.2, 2.3 and 9.0 mM for teniposide

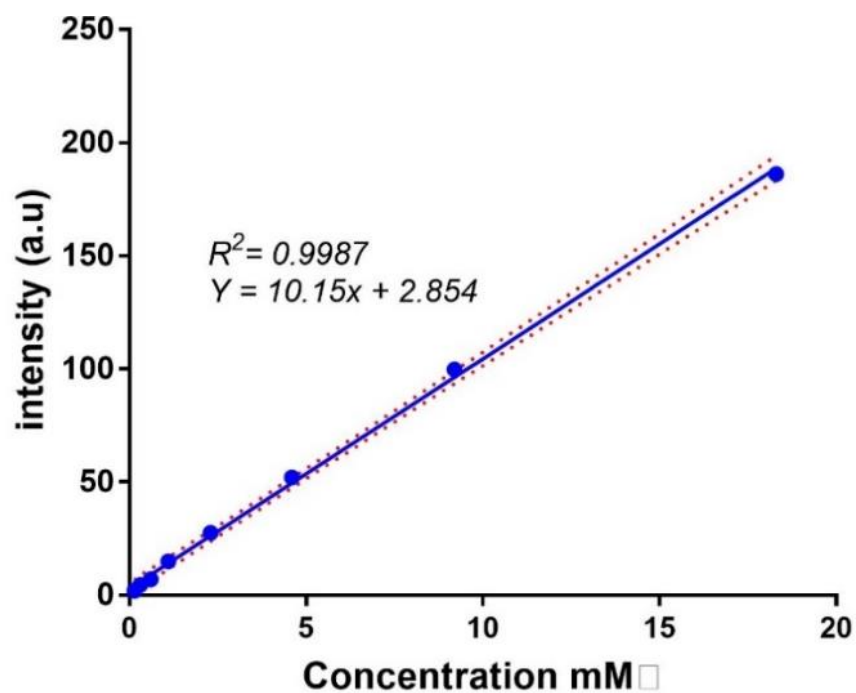


Figure 2-12 Standard curve for etoposide in 50:50 DCM: DMSO using fluorescence spectroscopy

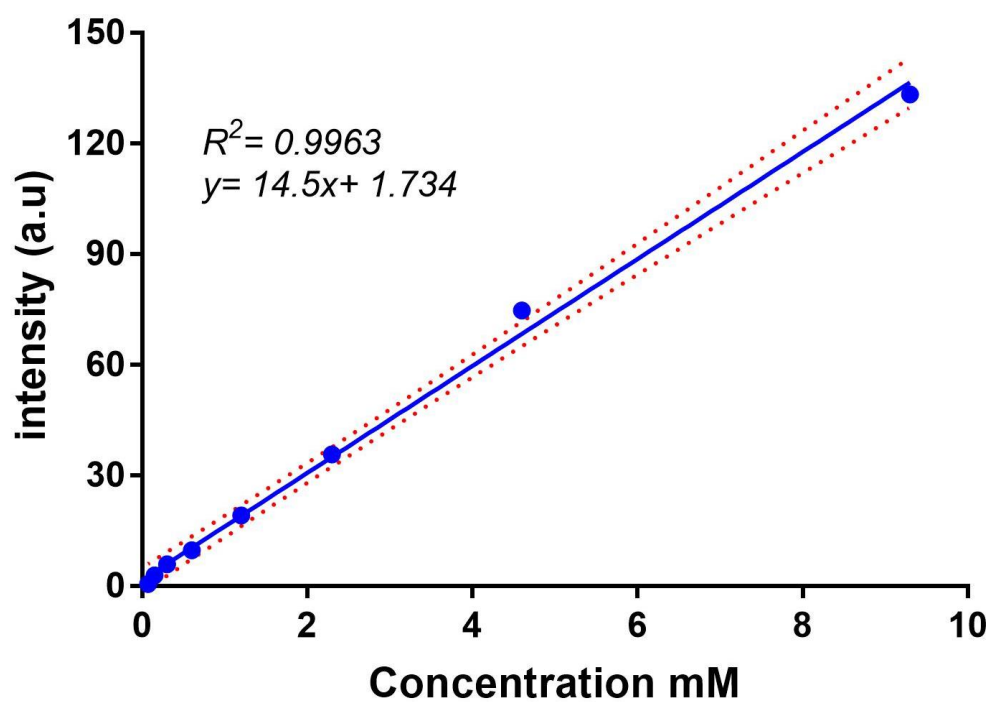


Figure 2-13 Standard curve for teniposide in 50:50 DCM: DMSO using fluorescence spectroscopy.

UV spectroscopy was used for the determination of etoposide phosphate at a wavelength of 290 nm. The etoposide, etoposide phosphate and teniposide incorporated into the nanoparticles were extracted as described in section 2.2.2.4. Also, to evaluate the robustness of the process, 100 μ M drug solution was prepared and used to spike empty nanoparticles, then analysed using fluorescence (etoposide and teniposide) and UV (etoposide phosphate). The mean percentage recovery obtained for etoposide, etoposide phosphate and teniposide were 101%, 97% and 95% respectively.

2.2.2.5 Drug release from nanoparticles

Nanoparticle suspensions (0.5 ml) were placed in dialysis tubing (regenerated cellulose, 10 KDa MWCO, 5 mm). The tube was placed in a release medium (50 ml) after sealing at both ends. The experiment was carried out under constant stirring at room temperature (25°C). Control samples of teniposide and etoposide from their separate DMSO stocks and Etoposide phosphate in PBS and empty nanoparticles spiked with a similar concentration of etoposide, etoposide phosphate, and teniposide were used to assess the diffusion kinetics of the drugs and account for interactions between nanoparticles and free drug within the dialysis bag. Samples (1 ml) were taken at 1 hour, 2 hours, 3 hours, 4 hours, 7 hours, 24 hours and 48 hours and replaced with fresh medium at 1,2,3,7 and 8 hours and the full medium was exchanged at 4 hours, 24 hours and 48 hours. The samples were stored at -20°C and thawed at 37°C then sonicated for 1 minute just before being taken for HPLC analysis.

2.2.3 Cytotoxicity experiments

2.2.2.1 Subculture

The cells (UW228-3) were subcultured every two days at about 80% confluence. To subculture, the medium was first aspirated out and the cells rinsed with PBS (5 ml). Trypsin EDTA (2.5 ml; 0.025% Trypsin in Ca^{2+} and Mg^{2+} free PBS solution) was added to the rinsed cells and incubated for 5 minutes until the cells were detached. Full medium, DMEM/F12 (10 ml) was added to the flask to stop the reaction of the trypsin and then transferred to a centrifuge tube. The cell suspension was centrifuged for 5 minutes at a speed of 13000 rpm. The supernatant was aspirated, and the cells left at the bottom. Full medium (10 ml) was added to the tube and the cell pellet was resuspended by mixing gently several times using a pipette. Cell suspension (1 ml) was transferred into a new flask and full medium (9 ml) was added and then incubated.

2.2.2.2 Determination of optimal numbers of cells per well

UW228-3 cells were trypsinized as described in section 2.2.2.1. After the cell suspension had been centrifuged, cells were diluted in 2 ml of serum free medium. Cell suspension (50 μl) was placed in a microcentrifuge tube and trypan blue (50 μl) was added. The mixture was thoroughly mixed. Trypan blue is a negatively charged chromophore which stains with damaged cell membrane. Living cells are not stained by trypan blue. A Pasteur pipette is then used to transfer a small amount of the trypan blue-cell solution on to the chamber on the haemocytometer.

The number of cells per ml of the cell suspension was calculated using the following equation

$$\frac{\text{Cells}}{\text{ml}} = \text{no of cells (per square)} \times \text{dilution factor} \times 10^4 \quad \text{Equation 12}$$

Dilutions were made such that the concentration of cells in wells were 2500, 5000, 7500 and 10000. Cell free medium was added to wells as control. Plates were incubated (37°C, 5% CO₂) for 24 hours, then the medium was removed from each well. MTT assay was carried out as detailed below.

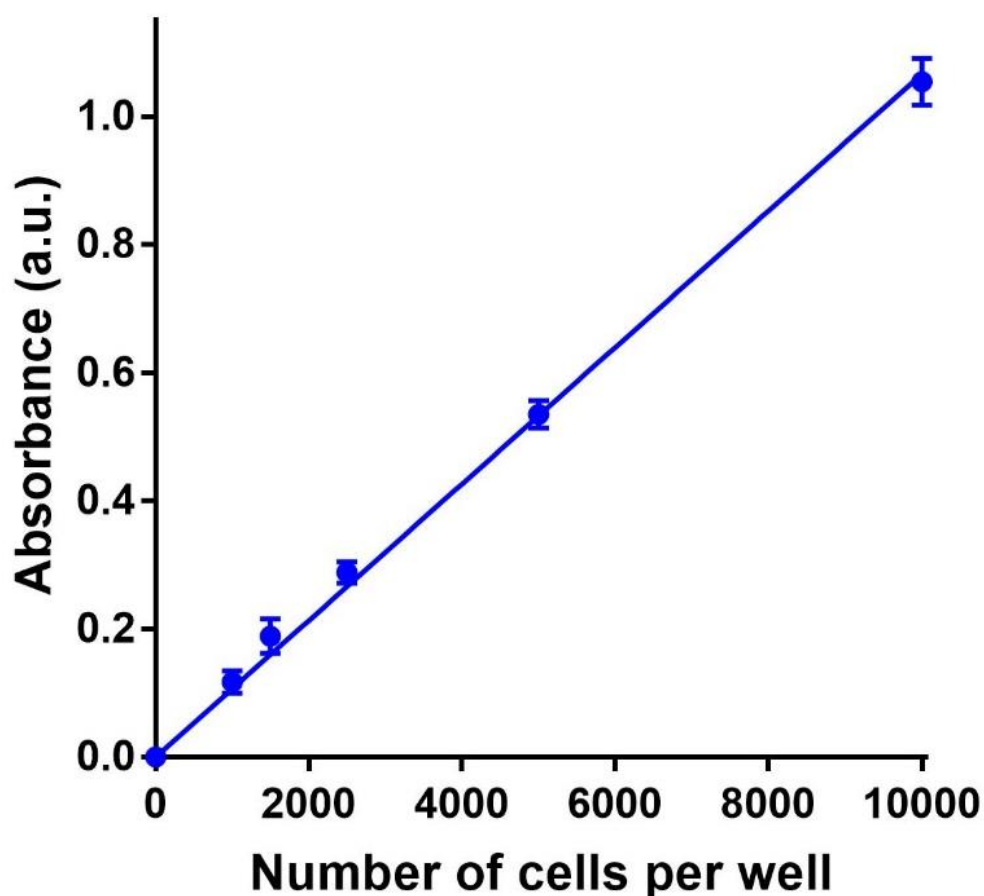


Figure 2-14 Graph of the number of cells per cell vs absorbance. A linear relationship was obtained up to 10000 cells. Data represents three determination form three plates; 3 replicates per plate for each dilution.

2.2.2.3 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

5000 cells/well of UW228-3 were seeded into 96 well plate and incubated in fully supplemented DMEM/F12 medium (150 µl). After 24 hours, the medium was removed and replaced with 150 µl of serum free medium containing treatment.

Free drugs: etoposide, etoposide phosphate and teniposide were prepared in serum free DMEM medium and added to the cells at defined concentration (0.01 µM – 300 µM) and incubated for 24 hours.

Nanoparticles: Freshly prepared nanoparticles were diluted with PBS (10x) solution to make an isotonic nanosuspension. The actual drug concentration determined as described in 2.2.2.4. Dilutions of the nanosuspensions were made in serum free DMEM medium to concentration of 1 nM to 200 nM.

After 24 hours incubation, the treatment was removed and DMEM medium (150 µl) was added followed by 15 µl of MTT reagent (5 mg/ml in PBS) per well was added and incubated for 4 hours. The media was discarded and DMSO (100 µl) was added and absorbance measured at 570 nm using a SpectraFluor spectrophotometer (Tecan Group Ltd Männedorf, Switzerland).

Separate wells containing 5000 Cells per well were treated with DMEM medium and Triton 0.2 %^{w/v} were used as a positive and negative control respectively. The percentage metabolic activity was calculated as the ratio of mean sample absorbance of triplicate readings with respect to mean absorbance of control wells using the following equation:

$$\text{Metabolic activity (\%)} = \left(\frac{S-T}{C-T} \right) * 100 \quad \text{Equation 13}$$

Where S is the absorbance obtained with the tested samples, T is the absorbance observed with Triton, and C is the absorbance observed with control.

2.2.4 Preparation of Polymer coated nanoparticles

2.2.4.1 Optimizing the preparation of polymer free drug nanoparticles

The first step was to determine the right time to add the polymer solution. This was done by monitoring particle size as the organic solvent containing the drug was mixed with the antisolvent.

2.2.4.2 Preparation of drug nanoparticles

The drug nanoparticles were first prepared and particle size determined. Etoposide or Teniposide) (2 mg) dissolved in acetone (2 ml) was mixed with water (4 ml) using a chamber mixer (Pharmacia, 50-60 Hz, 5 MPa) and collected in vials wrapped in aluminium foil.

The vials containing the resulting mixtures were left under magnetic stirring, in a fume hood to remove residual acetone. Small samples were taken at 0 hour, 4 hours and overnight to determine the particle size of drug nanoparticles as they formed.

2.2.4.3 Preparation of polymer coated drug nanoparticles

The method used here is based on the modification of the IDP method used by Abushrida (2011), for the preparation of polymer coated iron oxide nanoparticles (209). The method involved the use of a low ratio of polymer to drug to achieve a thin layer of coating. Previous exploratory work done

in the group has resulted in the successful coating of drugs by polymers. Assuming that a thin layer of coating of about 2 - 5 nm is to be placed on a nanoparticle of about 100 nm, calculations were done to determine the amount of polymer that would be needed.

Imagine a nanoparticle spherical in shape with a nanometer size of 100 nm (radius 50 nm) with a coating of 2 nm to 5 nm which will give a coated particle of about 104 nm (radius 52 nm) - 110 nm (radius 55 nm) (see Figure 2-15). The volume of the spheres can be calculated using the equation:

$$V = \frac{4}{3}\pi r^3$$

Equation 14

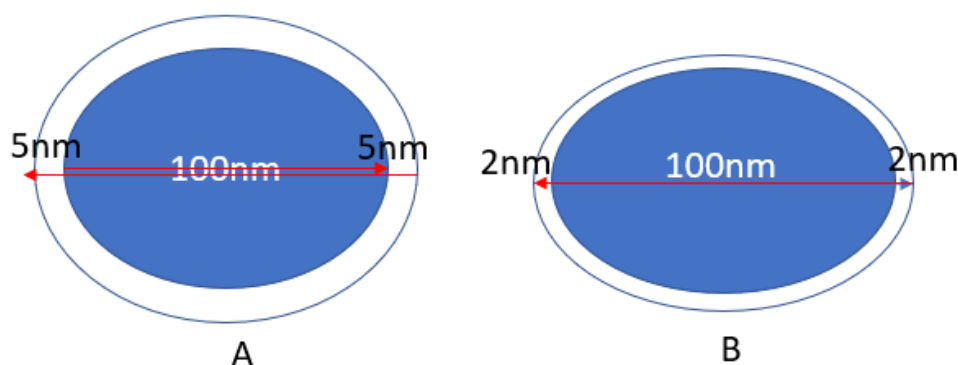


Figure 2-15 Illustration of a sphere with a thin coating showing the diameter of the inner sphere and the size of the polymer coating.

The volume of the inner sphere (representing the drug nanoparticle) was calculated to be 523,599 nm³ while the volume of the outer spheres A and B were calculated to be 696,910 nm³ and 588,977 nm³ respectively. From these calculations, the percentage polymer coating to achieve a thin 2 nm to 5 nm coatings for these drug nanoparticles would be between 12.5% - 33.1%. Assuming the density of the polymer and the drugs are close to equal, 0.3

mg and 0.5 mg (15% and 25% respectively) polymer for 2 mg of drug were the chosen starting point for the coating on this project.

Polymer (10 mg) was dissolved in acetone (2 ml), dilutions were made to prepare 2 ml solutions containing 0.3 mg and 0.5 mg polymer (see Figure 2-16). Each 2 ml polymer solution was added separately to a drug nanoparticle suspension in water prepared previously, using a chamber mixer (Pharmacia, 50-60 Hz, and 5 MPa) and collected in vials wrapped in aluminium foil.

The vials containing the resulting mixtures were left under magnetic stirring, overnight in a fume hood to remove residual acetone. The resulting nanoparticle suspensions were filtered using a 0.45 μm syringe filter before the separation of any unincorporated soluble drug.

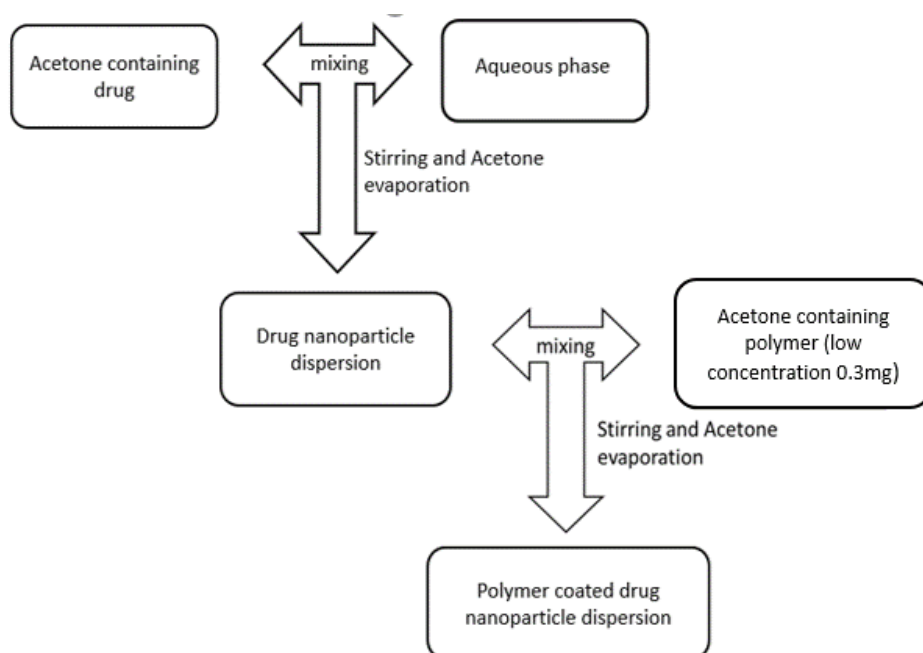


Figure 2-16 A simple flow chart of the process of preparation of the Polymer coated drug nanoparticles using a modified nanoprecipitation method. Low polymer concentration organic phase was used to ensure thin coating and high drug loading.

Chapter 3

3 Drug-polymer screening

3.1 Introduction

This chapter aims to study the interactions between the drugs (etoposide, etoposide phosphate, teniposide) and substituted PGA polymers. Acquiring this knowledge will be fundamental for choosing the appropriate polymer for each drug in order to make optimal drug loaded nanoparticle formulations. The advantages of nanoparticles and the extensive possibilities for drug delivery have been established (210,211). The success of the nanoparticles in cancer treatment, however, is dependent on a sufficient amount of drug getting into tumours and reaching all the cells (212). Specifically for local delivery to brain tumours, selectivity and prolonged tumour exposure is important for success.

Drug-polymer interactions have been studied, with the aim of trying to understand the complex processes that occur when drugs and polymers interact (213). Largely, the assumption in the literature is that hydrophobicity drives the interaction between drug and polymer. However, there are a lot of other types of interactions that could be occurring. The assembly of drug and polymer into nanoparticles has been said to be controlled by intermolecular interactions such as hydrogen bonding, hydrophobic-hydrophobic interactions and ionic interactions (214–216). These interactions have been shown to control nanoparticle properties such as drug release and loading, size and other physical properties (217). Jelonek *et al* observed strong interactions between Paclitaxel and PLA in paclitaxel loaded PLA micelles (218). They obtained FTIR results showing strong interactions between the NH and OH groups of Paclitaxel and PLA. They concluded that these interactions were

responsible for the higher drug loading efficiency and slower release results obtained. The overall contribution of all the different interactions will be key to drug incorporation and release. Solubility parameters is a tool that has been widely used to predict polymer-drug compatibility, however recent work in our group showed this to be unreliable. Turpin *et al* showed the inadequacies of predicting drug-polymer interactions using computational methods based on Hildebrand and Hansen solubility parameters and determination of the Flory– Huggins parameter (219). They demonstrated the need to assess polymer drug interactions by experimental determination.

The role of the polymer to the success of the NPDS emphasizes the importance of a more systematic approach when matching a polymer to a drug. The matches can be made based on the observed interactions between the drugs and polymers. Determination of drug release from nanoparticles is very time consuming and slow. However, using solid dispersion in thin films permits a higher throughput approach. Solid dispersions are made up of drugs dispersed in polymeric carriers and they have provided a means to study drug-polymer interactions (82,213,220). Some of the techniques commonly used in studying drug-polymer interactions include Infrared and Raman spectroscopies, Differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR) (84,88,213).

The novel approach of this project is to consider a set of polymers with different physicochemical properties and assess their interaction with drugs through their ability to retain the drugs in release experiments and to assess some of these possible interactions using contact angle and FTIR measurements. Furthermore, the direct implications of this affinity and drug retention to properties of nanoparticles such as drug loading and release will be studied.

3.1.1 Aims of chapter 3

The underlying experimental determination is based on the idea that the polymers with the most affinity will retain the drugs more and have improved drug loading. This hypothesis is based on the knowledge that the way a polymer interacts with a particular drug is not predictive of its reaction with other drugs (221). Hence, the need for a guided and evidence-based polymer selection for drugs.

This chapter, therefore, screens three drugs against a set of chosen modified polymers. The screening was carried out by preparing drug-polymer films and then monitoring interactions of the drug and polymer within the films. The focus was to carry out a thorough screening of three drugs against nine different PGA polymers with substituted functional groups. These functional groups confer properties to these polymers which are different from the unmodified polymer backbone.

The projected endpoint is to prepare nanoparticles that would incorporate more drug and retain the drug for a longer period.

1. Firstly, the aim is to monitor drug retention as a measure of the changing interactions between drugs and polymers. It is expected that the diffusion of a drug out a thin film would be rapid. Based on our hypothesis, interactions between the drug and polymer would slow down the diffusion rate, hence increase retention. The release experiments were carried out to observe any deviations to the expected rapid release.
2. Secondly, to Carry out FTIR and contact angle measurements to provide possible explanations to the results from the release experiments. These changes in contact angle and the FTIR were to

provide clarity to the release results or to show the need for further explanations.

3.2 Materials and methods

3.2.1 Release experiments

Release experiments from 40%^w/_w drug-polymer films were carried out as described in Section 2.2.1.1.1.

3.2.2 Fourier transform infrared

The drug-polymer films were examined for changes in wavenumber of functional groups as described in section 2.2.1.2.

3.2.3 Contact angle

Contact angle measurements of drug, drug-polymer films and polymer were carried out as described in section 2.2.1.3.

3.2.4 Data Analysis

The experiments were performed at least three times and the results obtained were analysed (including statistical) using excel and graph-pad prism 7.0. The drug release after 24 hours was calculated from the concentrations obtained from HPLC and plotted against time.

3.3 Results

3.3.1 Release experiments

The first set of experiments were carried out to determine the retention of drugs by the nine different polymers which should be an indication of the interaction between the drugs and polymers. The release experiments were carried out by a dialysis method in which the drug-polymer films containing etoposide and teniposide were placed in 1% sodium cholate in PBS (10 ml) and those containing etoposide phosphate were placed in PBS (5 ml) to maintain sink conditions as explained in section 2.2.1.1.1. Each drug – polymer film contained 212 µg of drug and 318 µg of polymer. A control containing drug alone (212 µg) was used for all drugs to monitor the normal behaviour of the drug in the absence of polymer.

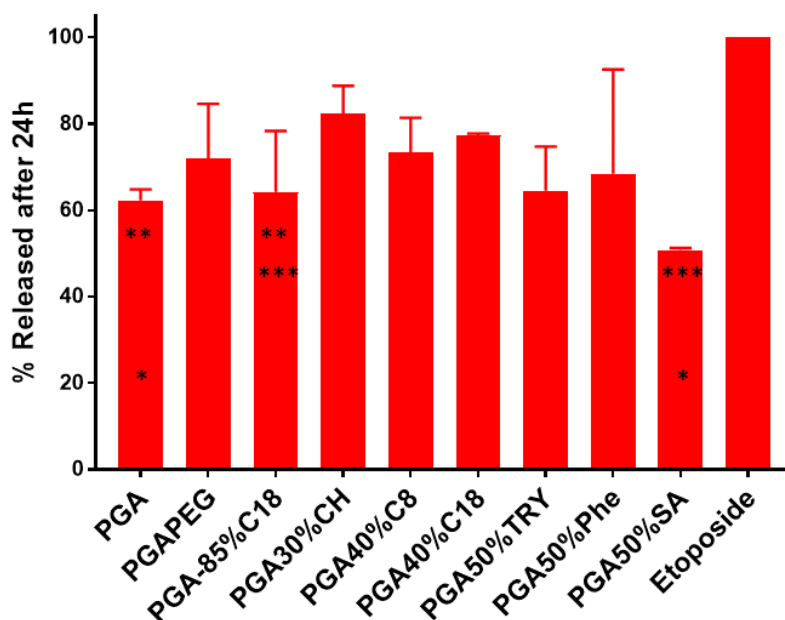


Figure 3-1 Plot of the percentage of etoposide released from the different polymers after 24 hours. Error bars show the standard deviation of results from three separate experiments. An unpaired t-test was carried out to check the statistical significance of some of the key results $p^*=0.0243$, $p^{**}=0.7664$ and $p^{***}=0.3111$

Figure 3-1 is a plot of the release of etoposide from etoposide loaded drug-polymer films after 24 hours. The diffusion of free etoposide (in the absence of polymer) monitored in the same way as the drug-polymer films. After 24 hours the free etoposide was fully dispersed in the dissolution media. For the drug-polymer films, the majority of the release of etoposide from the polymers was over 60% and relatively variable with about 60 – 80% release observed from most of the polymers. The PGA50%SA was notable as it has released a lower amount than the other polymers (about 51%), a statistically significant difference was observed between the release from this polymer and the PGA backbone. PGA85%C18, PGA50%TRY and PGA were next in terms of percentage drug release of a little above 60%. However, no statistically significant difference was observed between the release from PGA85%C18 (from this group) and PGA or PGA50%SA.

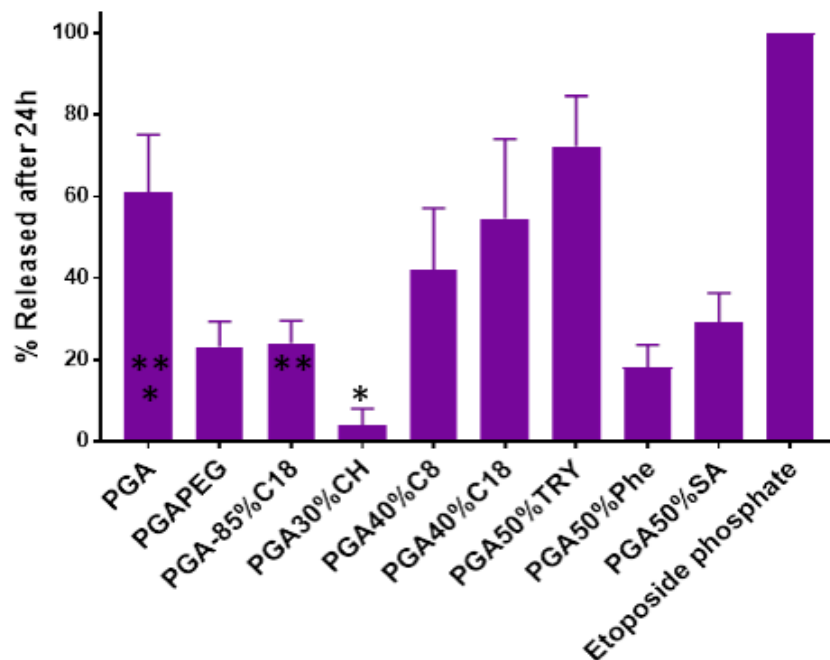


Figure 3-2 A plot of the percentage of etoposide phosphate released from the different polymers after 24 hours. Error bars show the standard deviation of results from three separate experiments. An unpaired t-test was carried out to check the statistical significance of some of the key results $p^*=0.0313$, $p^{**}=0.0742$

Figure 3-2 is a plot of the release of etoposide phosphate from etoposide phosphate loaded drug-polymer films after 24 hours. Similar to the free etoposide, after 24 hours the free etoposide phosphate was fully dispersed in the dissolution media. In general, the amount of etoposide phosphate released from the polymers appeared to be less than etoposide. Four polymers gave a release of between 40% to about 70%. A second group of polymers showed a release of about 18 – 30%. PGA_{30%}CH released an exceptionally low amount of etoposide phosphate. The release was significantly different from the release from the PGA backbone.

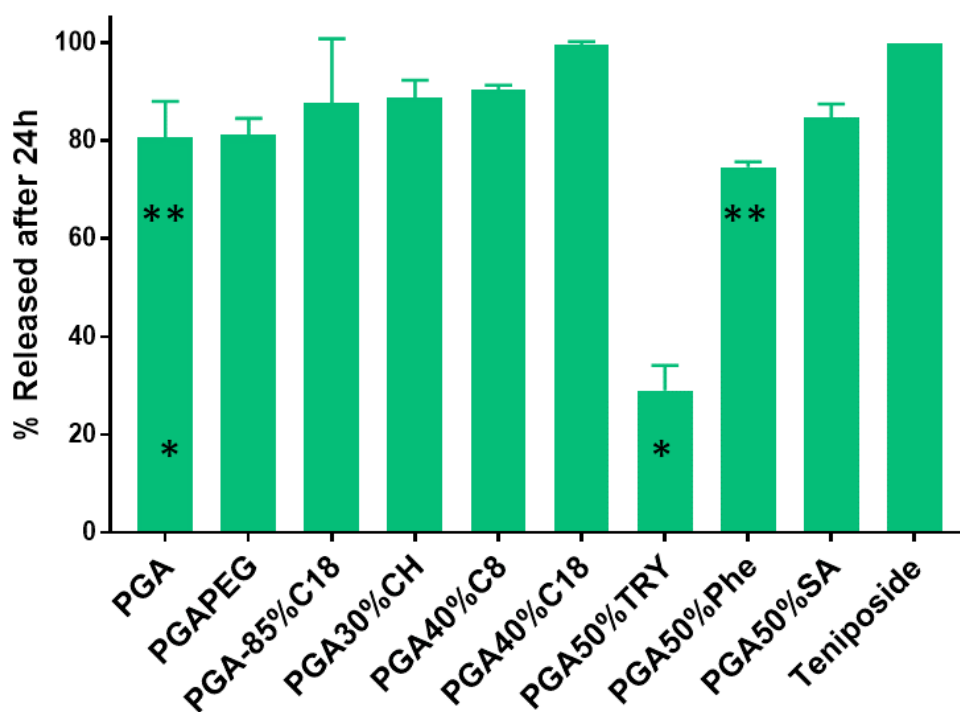


Figure 3-3 A plot of the percentage of teniposide released from the different polymers after 24 hours. Error bars show the standard deviation of results from three separate experiments. An unpaired t-test was carried out to check the statistical significance of some of the key results $p^*=0.0151$, $p^{**}=0.3803$

Looking at Figure 3-3, in general, most of the release of Teniposide was above 80%. PGA_{50%}Phe released a slightly lower amount though not

statistically significantly different from PGA. PGA50%TRY released a considerably lower amount of teniposide, the release, in this case, was statistically different from PGA.

3.3.2 Fourier transform infrared spectroscopy (FTIR)

FTIR was the first experiment carried out to attempt to explain the drug retention by each film and to further see if the changes were from interactions due to bond formation or changes to the functional groups present. The free drugs, empty films and the drug-polymer films were analysed using ATR FT-IR. Figure 3-4 shows the spectrum obtained for the polymer backbone PGA. The spectrum shows a broad O-H peak at 3450 cm^{-1} , a peak at $2950\text{--}2850\text{ cm}^{-1}$ representative of the alkyl C-H bond. The stretching C=O bond of the carbonyl peak appeared at 1731 cm^{-1} , the C-O-C stretching peak appeared at 1095 cm^{-1} and the bending O-H bond of free primary and tertiary alcohol at $1090\text{--}1040\text{ cm}^{-1}$.

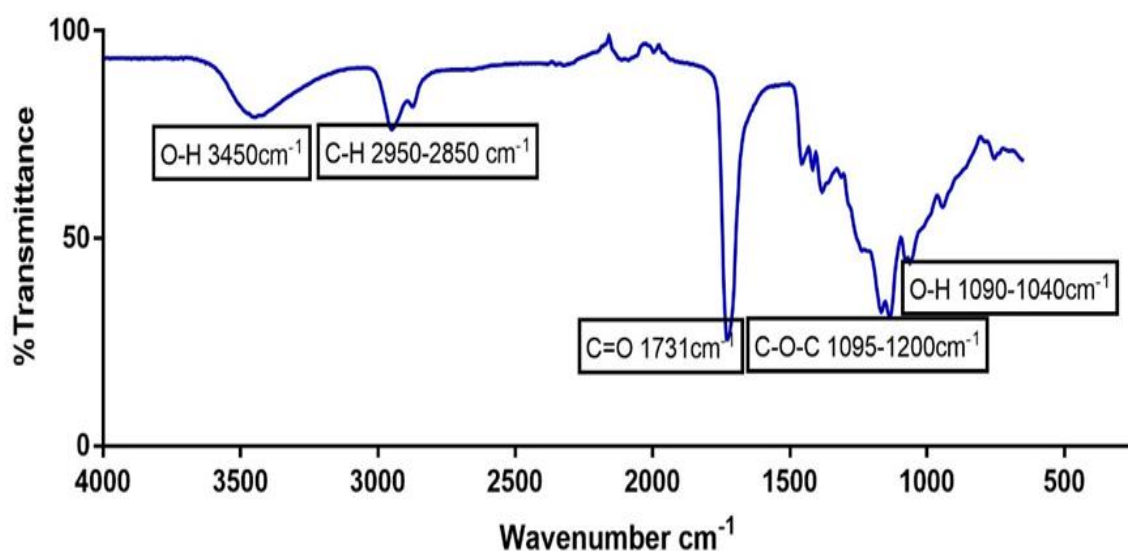


Figure 3-4 ATR-FTIR spectra of PGA in the spectral range of between 4000 and 650 cm^{-1} showing peaks for OH, CH, C=O, C-O-C and O-H of free primary and tertiary alcohols at 3450 cm^{-1} , $2950\text{--}2850\text{ cm}^{-1}$, 1731 cm^{-1} , $1095\text{--}1200\text{ cm}^{-1}$ and $1090\text{--}1040\text{ cm}^{-1}$ respectively.

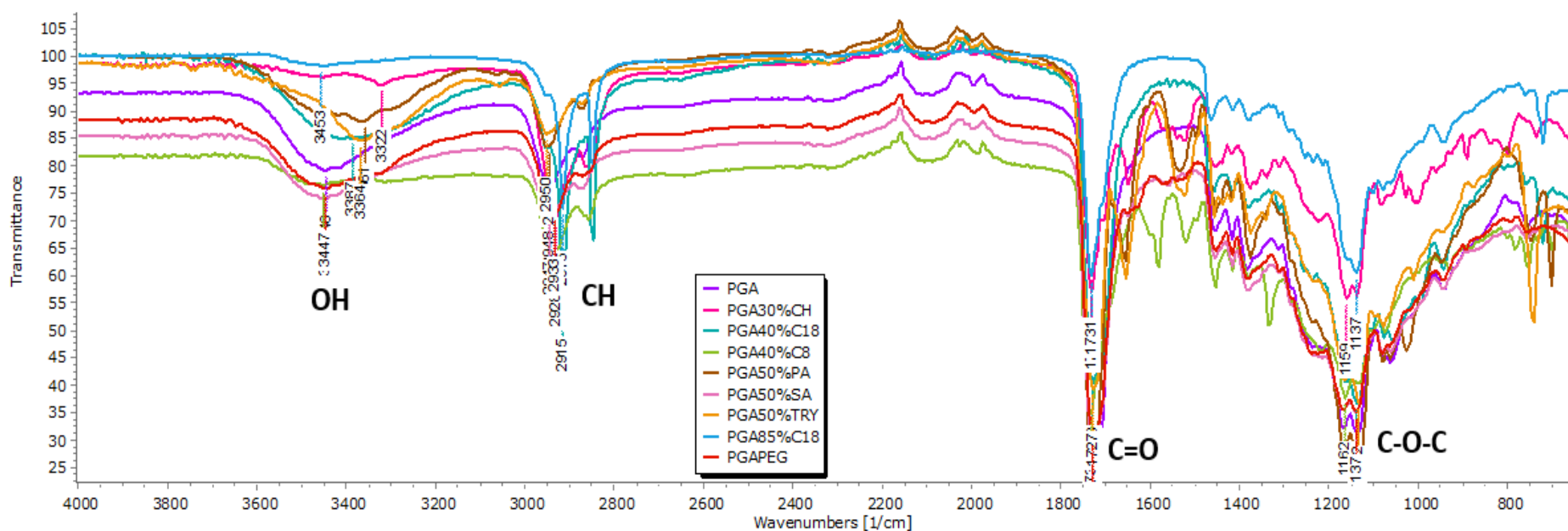


Figure 3-5 ATR FT-IR spectra of all the polymers showing the peaks assigned to the functional groups present. Showing the shift in the wavenumbers where the stretching of the O-H, C-H, C=O and C-O-C bonds occur due to OH substitution in the PGA backbone.

The substituted polymer spectra showed similar shifts in wavenumbers, ranging from as low as 5 cm^{-1} – 100 cm^{-1} (Figure 3-5). These shifts are due to the reduction in the number of free OH groups due to varying degree of substitution. In addition, PGA50%Phe contains indole with an NH peak stretching at 3406 cm^{-1} . Also, as secondary amines PGA50%Phe and PGA50%TRY have NH peak stretching at $3350 - 3310\text{ cm}^{-1}$ and an NH wag at $910-665\text{ cm}^{-1}$

Looking at Figure 3-6, the spectra obtained for etoposide, etoposide phosphate and teniposide showed peaks for O-H bonds at between 3300 and 3450 cm^{-1} , peaks at $2919 - 2921\text{ cm}^{-1}$ representative of the alkyl C-H bond. The C=O bond of the carbonyl peak appeared at between $1759-1774\text{ cm}^{-1}$, the backbone of the compounds contains aromatic phenyl rings which have stretching C=C bond with peaks appearing at between $1500-1700\text{ cm}^{-1}$. Changes to the wavenumber of the peaks of the functional groups in the film will be used as evidence of interactions the polymers and the drugs.

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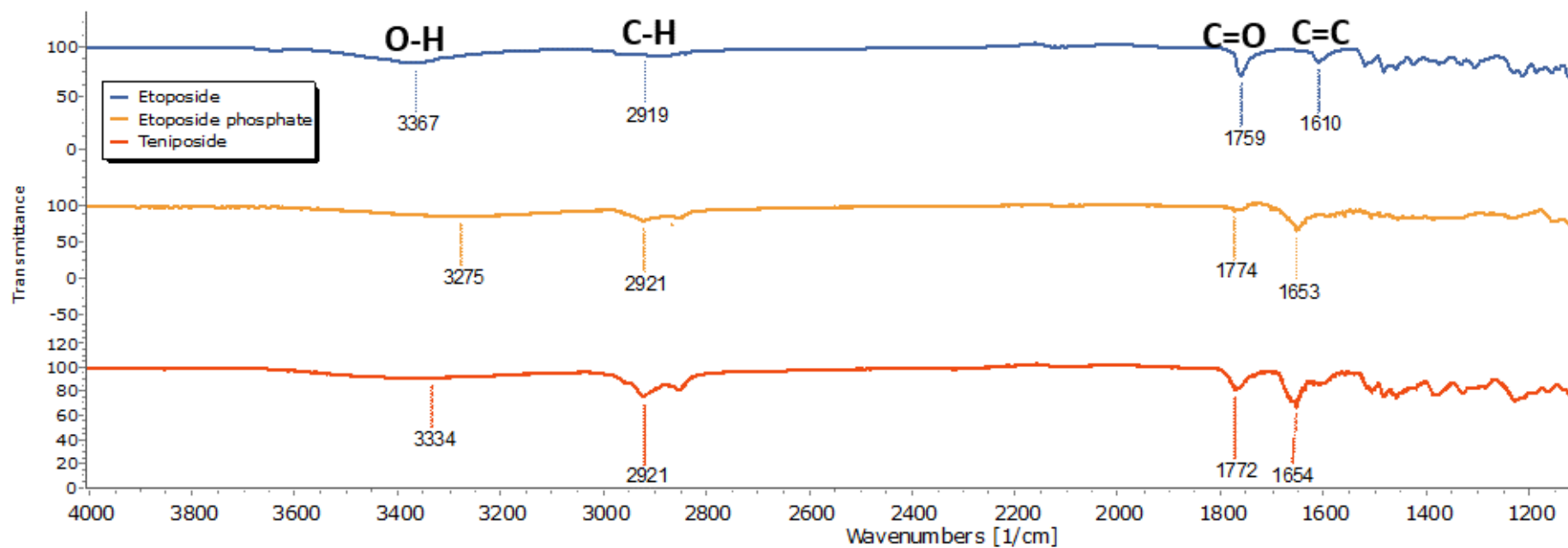


Figure 3-6 ATR FT-IR spectra of etoposide, etoposide phosphate and teniposide showing the assigned peaks of the bonds present.

The spectra obtained for the drug-polymer films showed peaks as explained below. Peaks appeared for O-H bonds, at between 3200 and 3450 cm^{-1} , at 2911 – 2921 cm^{-1} representative of the alkyl C-H bond, at 1759-1777 cm^{-1} for C=O bond of the carbonyl peak and at the 1500-1700 cm^{-1} for the stretching C=C bond of the aromatic backbone. Looking at all the films' FTIR spectra, there were changes to the broad O-H and the carbonyl peak positions in the films compared to individual drugs. The specific changes as they apply to each drug and their respective films will be discussed further.

3.3.2.1 PGA Films

The drug loaded PGA films showed the most significant changes in the position of the broad O-H peak (see Figure 3-7). This peak appeared at 3212 cm^{-1} for Etoposide-PGA film, at 3455 cm^{-1} for Etoposide phosphate-PGA films and 3449 cm^{-1} for the Teniposide-PGA film. The peak of the C-H bond changed only slightly across the drug loaded PGA films. For the carbonyl (C=O) bond, small changes occurred for the etoposide and teniposide loaded PGA films. In the etoposide phosphate-PGA film, the carbonyl peak relating to the disappeared from 1774 cm^{-1} . A new carbonyl (C=O) peak showed at 1716 cm^{-1} for etoposide phosphate loaded PGA-PEG film, and 1731 and 1735 cm^{-1} for both etoposide and teniposide loaded PGA-PEG films respectively. The peak is similar to that which was observed at 1725 cm^{-1} in empty PGA.

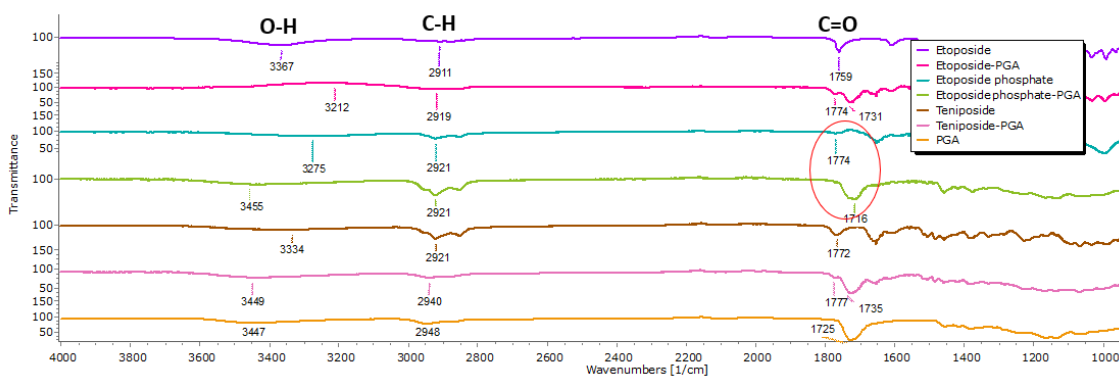


Figure 3-7 ATR FT-IR spectra of drug loaded PGA films showing the assigned peaks of the bonds and the changes in the films' spectra. The red circle shows where a peak that appears in the drug spectrum disappears in the film.

3.3.2.2 PGA-PEG Films

Looking at Figure 3-8, the broad O-H peak of Etoposide-PGA-PEG film appeared at 3241 cm^{-1} , while for Etoposide phosphate-PGA-PEG film a slight change occurred in the peak position and it appeared at 3263 cm^{-1} . In the spectrum of the Teniposide-PGA-PEG film, the peak appeared at 3449 cm^{-1} , which is closer to where it appears in the polymer. The peak of the C-H bond changed only slightly across the drug loaded PGA-PEG films. Small changes occurred in the carbonyl (C=O) peak positions for the etoposide and teniposide loaded PGA films. While in the etoposide phosphate-PGA-PEG film, the carbonyl peak disappeared from 1774 cm^{-1} . There is a separate C=O peak at 1719 cm^{-1} at etoposide and etoposide phosphate loaded PGA-PEG films and 1720 cm^{-1} for teniposide-PGA-PEG similar to the peak at 1726 cm^{-1} in PGA-PEG.

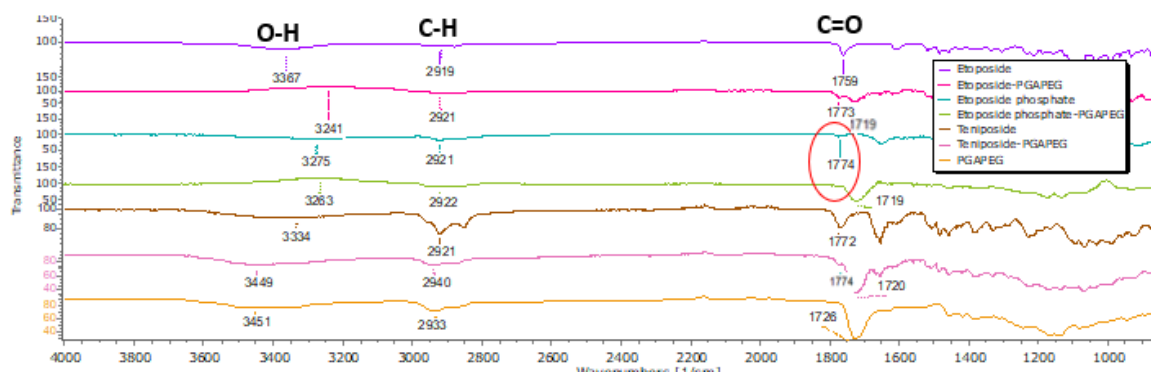


Figure 3-8 ATR FT-IR spectra of drug loaded PGA-PEG films, drugs and PGA-PEG showing the assigned peaks of the bonds and changes in the films' spectra. The red circle shows where a peak that appears in the drug spectrum disappears in the film.

3.3.2.3 PGA40%C8 Films

The spectra of the drug loaded PGA₄₀%C8 films showed the most significant changes in the broad O-H peak (see Figure 3-9). This peak appeared at 3266 cm⁻¹, 3346 cm⁻¹, and 3450 cm⁻¹ for Etoposide-PGA₄₀%C8, Etoposide phosphate-PGA₄₀%C8 and Teniposide-PGA₄₀%C8 films respectively. The peak of the C-H bond changed only slightly across the drug loaded PGA₄₀%C8 films. The carbonyl (C=O) peak position disappeared in the etoposide phosphate-PGA₄₀%C8 film. The new carbonyl C=O peak similar to the peak at 1734 cm⁻¹ occurred at 1731 - 1734 cm⁻¹ in all the drug loaded PGA₄₀%C8 films.

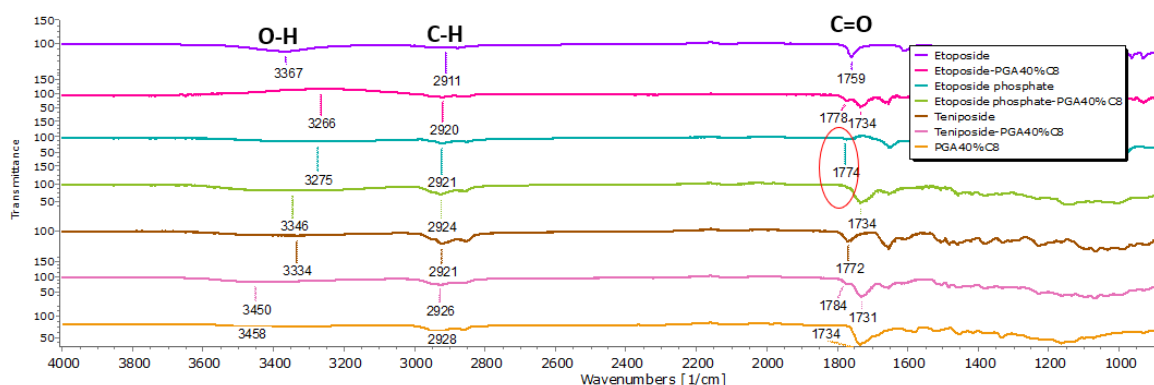


Figure 3-9 ATR FT-IR spectra of drug loaded PGA40%C8 films, drugs and PGA showing the assigned peaks of the bonds and changes in the films' spectra. The red circle shows where a peak that appears in the drug spectrum disappears in the film.

3.3.2.4 PGA40%C18 Films

Key changes in the PGA40%C18 occurred in the broad OH peak and the C=O peak (see Figure 3-10). The broad O-H peak appeared at 3446 cm^{-1} , 3410 cm^{-1} and 3444 cm^{-1} for Etoposide-PGA40%C18, Etoposide phosphate-PGA40%C18 and Teniposide-PGA40%C18 films respectively. In the case of the C=O peak, it disappeared in the Etoposide phosphate-PGA40%C18 and Teniposide-PGA40%C18 films. A new carbonyl C=O appears in the films just as in the polymers at different wavenumbers. At 1736 cm^{-1} , 1710 cm^{-1} and 1741 cm^{-1} for Etoposide-PGA40%C18, Etoposide phosphate-PGA40%C18 and Teniposide-PGA40%C18 films respectively.

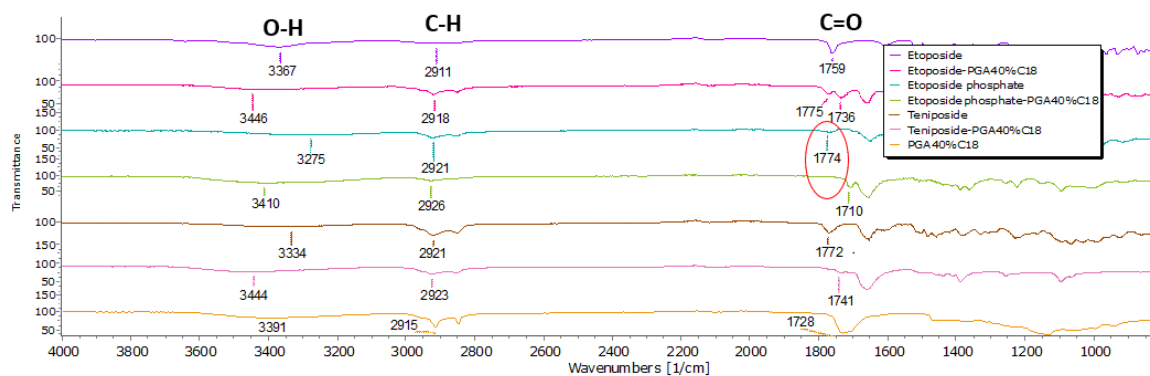


Figure 3-10 ATR FT-IR spectra of drug loaded PGA40%C18 films, drugs and PGA showing the assigned peaks of the bonds and changes in the films' spectra. The red circle shows where a peak that appears in the drug spectrum disappears in the film.

3.3.2.5 PGA85%C18 Films

The broad OH peak showed changes in the etoposide-PGA85%C18 and etoposide phosphate-PGA85%C18 films (see Figure 3-11). The new wavenumbers were 3330 cm^{-1} and 3345 cm^{-1} respectively compared to the OH peak positions in the drug. In teniposide-PGA85%C18, only a 1 cm^{-1} shift was observed. In the case of the C=O peak, a shift in the wavenumber from 1769 in the drug to 1775 in the etoposide-PGA85%C18. It disappeared in the Etoposide phosphate-PGA40%C18 and Teniposide-PGA40%C18 films. There was no new carbonyl peak in the etoposide-PGA85%C18 film. However, in the Etoposide phosphate-PGA40%C18 and Teniposide-PGA40%C18 films, the new carbonyl C=O peaks appeared at 1734 cm^{-1} and 1736 cm^{-1} respectively.

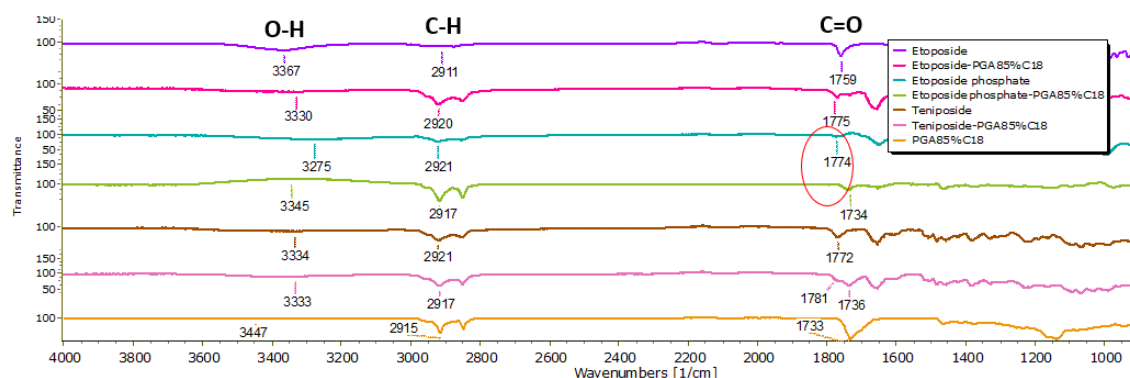


Figure 3-11 ATR FT-IR spectra of drug loaded PGA85%Cl8 films, drugs and PGA showing the assigned peaks of the bonds and changes in the films' spectra. The red circle shows where a peak that appears in the drug spectrum disappears in the film.

3.3.2.6 PGA30%CH Films

The observed changes in the PGA30%CH films as seen in Figure 3-12 are as follows. The OH peak disappeared in the Etoposide-PGA30%CH and teniposide-PGA30%CH films. In contrast, the carbonyl peak shifted to 1783 cm^{-1} and 1758 cm^{-1} respectively. The etoposide phosphate-PGA30%CH film showed a shift in the OH broad peak to 3325 cm^{-1} and the carbonyl C=O peak disappeared in this film. None of the films showed a new C=O peak in their spectra.

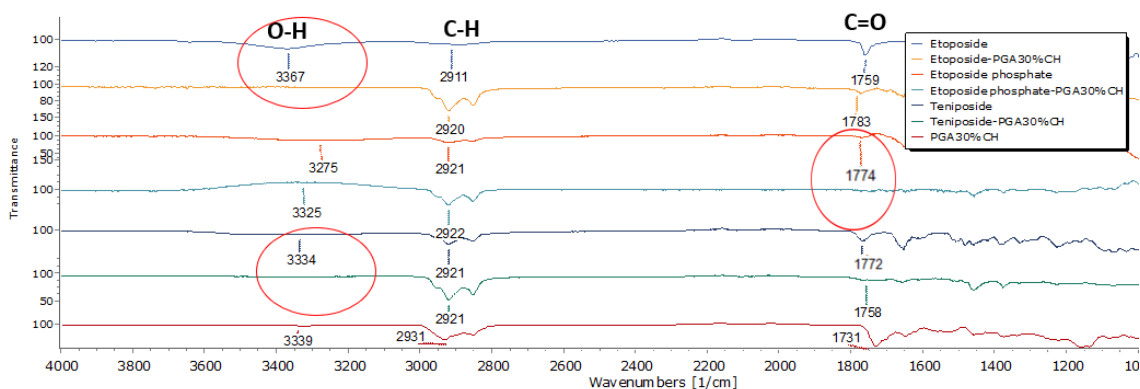


Figure 3-12 ATR FT-IR spectra of drug loaded PGA30%CH films, drugs and PGA showing the assigned peaks of the bonds and changes in the films' spectra. The red circles show where a peak that appears in the drug spectrum disappears in the film.

3.3.2.7 PGA50%SA Films

In the PGA50%SA films (Figure 3-13), the broad OH bond and the carbonyl peaks both disappeared in the etoposide phosphate-PGA50%SA film. Shifts were observed in the OH peak in etoposide-PGA50%SA and teniposide-PGA50%SA to 3411 cm^{-1} and 3444 cm^{-1} . However, for the carbonyl peaks, a shift of only 2 cm^{-1} was observed for etoposide-PGA50%SA and no change at all for teniposide-PGA50%SA. The new carbonyl C=O peak appeared at 1737 cm^{-1} , 1734 cm^{-1} and 1725 cm^{-1} for etoposide-PGA50%SA, etoposide phosphate-PGA50%SA and teniposide-PGA50%SA films respectively.

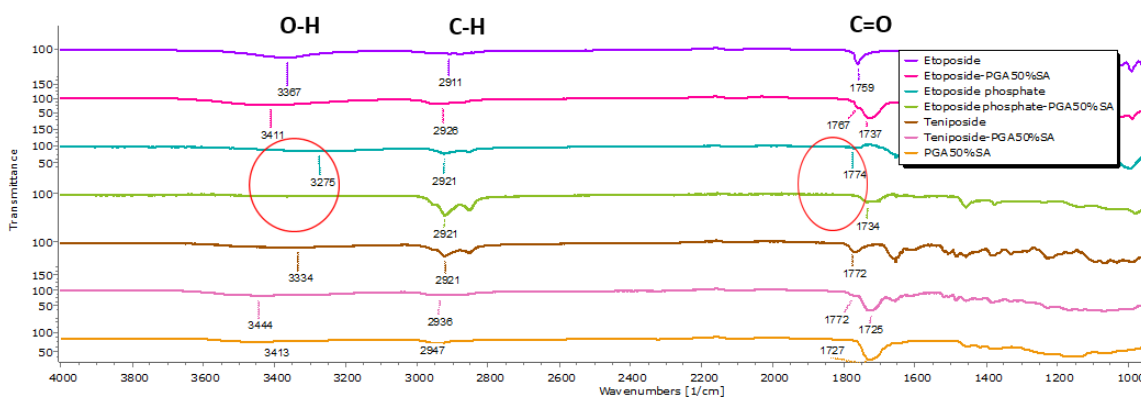


Figure 3-13 ATR FT-IR spectra of drug loaded PGA50%SA films, drugs and PGA showing the assigned peaks of the bonds and changes in the films' spectra. The red circles show where a peak that appears in the drug spectrum disappears in the film.

3.3.2.8 PGA50%Phe Films

The changes observed in the PGA50%Phe films compared to the drugs were in the stretching broad OH and C=O peaks (see Figure 3-14). The OH peak shifted to 3318 cm^{-1} in both etoposide-PGA50%Phe and etoposide phosphate-PGA50%Phe films, and 3319 cm^{-1} in teniposide-PGA50%Phe film. The carbonyl peaks shifted to 1780 cm^{-1} in both etoposide-PGA50%Phe and teniposide-PGA50%SA films, but completely disappeared in etoposide phosphate-PGA50%Phe film. A new carbonyl peak C=O appeared at 1735 cm^{-1} , 1734 cm^{-1} and 1737 cm^{-1} in etoposide-PGA50%Phe and etoposide phosphate-PGA50%Phe films and teniposide-PGA50%Phe films respectively.

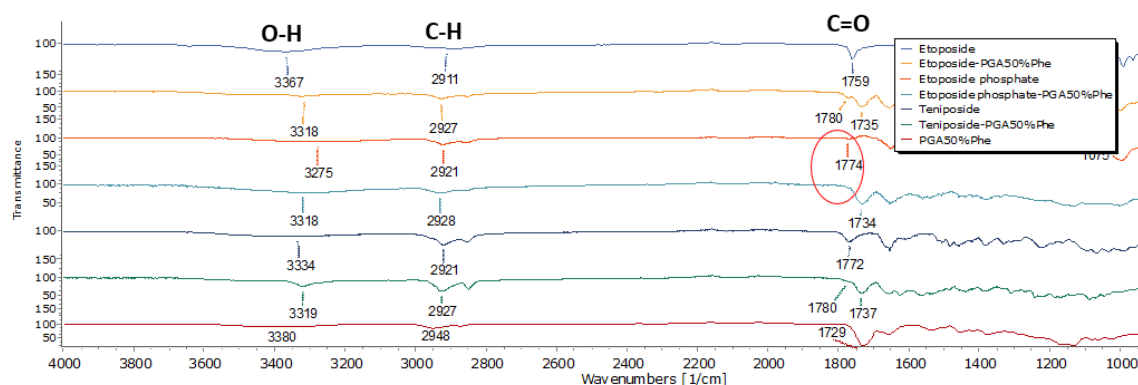


Figure 3-14 ATR FT-IR spectra of drug loaded PGA50%Phe films, drugs and PGA showing the assigned peaks of the bonds and changes in the films' spectra. The red circle shows where a peak that appears in the drug spectrum disappears in the film.

3.3.2.9 PGA50%TRY Films

The observed peak position changes in the PGA50%TRY films were mostly in the broad OH peak (see Figure 3-15). The peak disappeared in the Etoposide-PGA50%TRY, etoposide phosphate-PGA50%TRY and teniposide-PGA50%TRY films. In the case of the carbonyl peak, small shifts were observed in the etoposide phosphate-PGA50%TRY and teniposide-PGA50%TRY of 4 and 2 cm^{-1} . The peak disappeared in Etoposide-PGA50%TRY. The carboxylic acid C=O peaks were observed at 1723 cm^{-1} , 1725 cm^{-1} and cm^{-1} 1720 respectively for etoposide-PGA50%TRY, etoposide phosphate-PGA50%TRY and teniposide-PGA50%TRY films respectively.

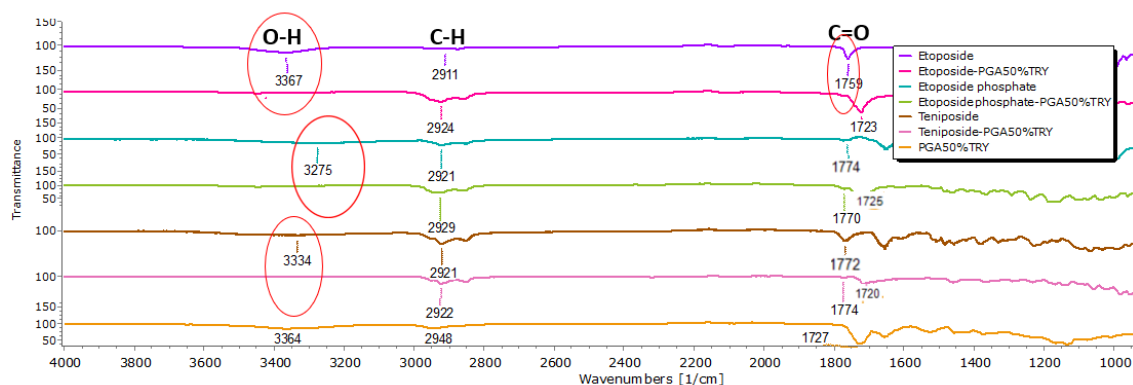


Figure 3-15 ATR FT-IR spectra of drug loaded PGA50%TRY films, drugs and PGA showing the assigned peaks of the bonds and changes in the films' spectra. The red circles show where a peak that appears in the drug spectrum disappears in the film.

Figure 3-16, Figure 3-17 and Figure 3-18 summarise the changes in wavenumbers of the O-H and carbonyl peaks of the etoposide, etoposide phosphate and teniposide loaded films spectra. The biggest changes seen for etoposide (Figure 3-16) were in the PGA50%TRY where the O-H and carbonyl peak were no longer present. Overall the OH peak appeared to show bigger wavenumber changes than the carbonyl. PGA30%CH film spectrum has a missing OH peak. PGA and PGA-PEG showed highest wavenumber changes followed by PGA40%C8 and PGA40%C18. The carbonyl groups showed small changes overall except PGA50%TRY.

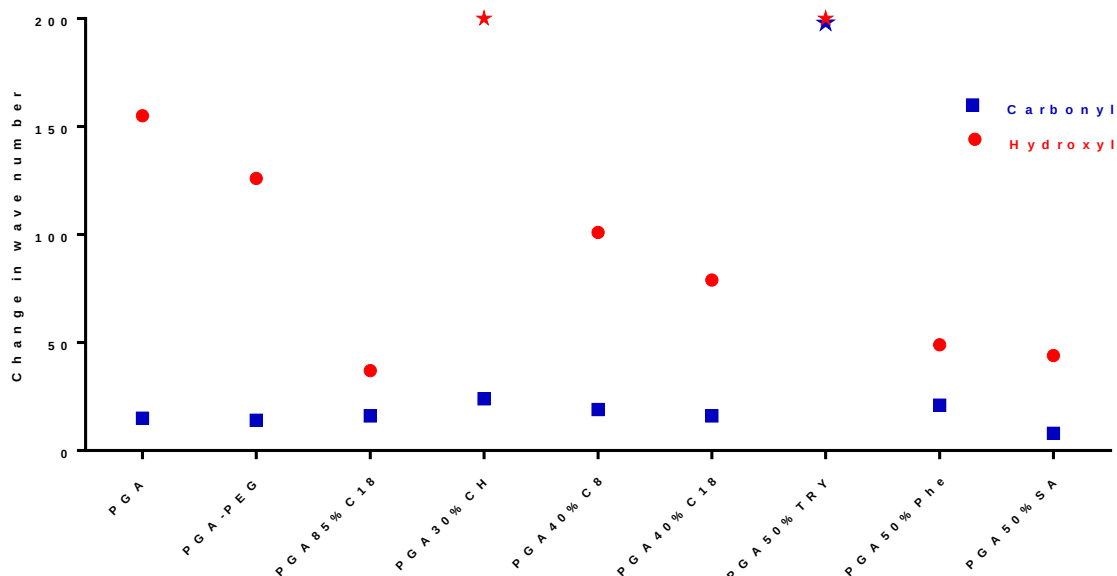


Figure 3-16 A plot of the change in wavenumber (cm^{-1}) of the o-H and carbonyl bond peaks observed in etoposide loaded drug-polymer films. The red and blue stars represent the drug-polymer films spectra with missing O-H and carbonyl peaks respectively.

For etoposide phosphate (Figure 3-17) all the drug-polymer films spectra had missing carbonyl peaks except PGA50%TRY which has a very small change in wavenumber. The OH peaks were not present in the PGA50%TRY and PGA50%SA films. PGA showed a very high change in wavenumber for the OH peak followed by PGA40%C18. They are followed by the group of PGA85%C18, PGA40%C8, PGA30%CH and PGA50%Phe which had similar changes in wavenumber. PGA-PEG has the lowest OH peak change in wavenumber.

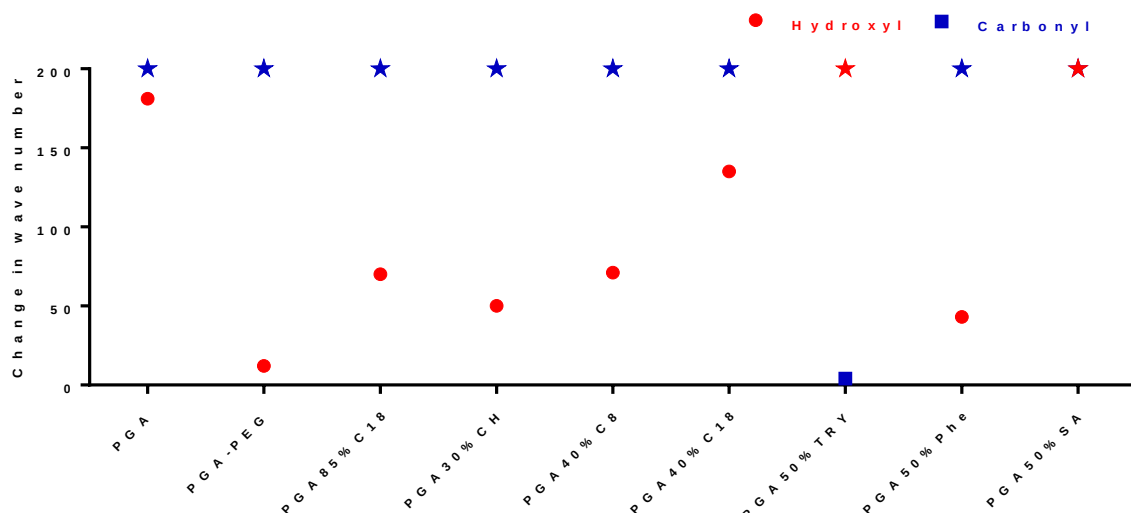


Figure 3-17 A plot of the change in wavenumber (cm^{-1}) of the o-H and carbonyl bond peaks observed in etoposide phosphate loaded drug-polymer films. The red and blue stars represent the drug-polymer films spectra with missing O-H and carbonyl peaks respectively.

The teniposide-polymer films (Figure 3-18) showed only small or no changes in the carbonyl peak except for PGA40%C18 which had a missing carbonyl peak. PGA30%CH and PGA50%TRY films spectra had missing OH peaks. The rest of the teniposide polymer films spectra had similar changes in wavenumber of the OH peak except PGA85%C18 and PGA50%Phe films with very little changes in the wavenumber.

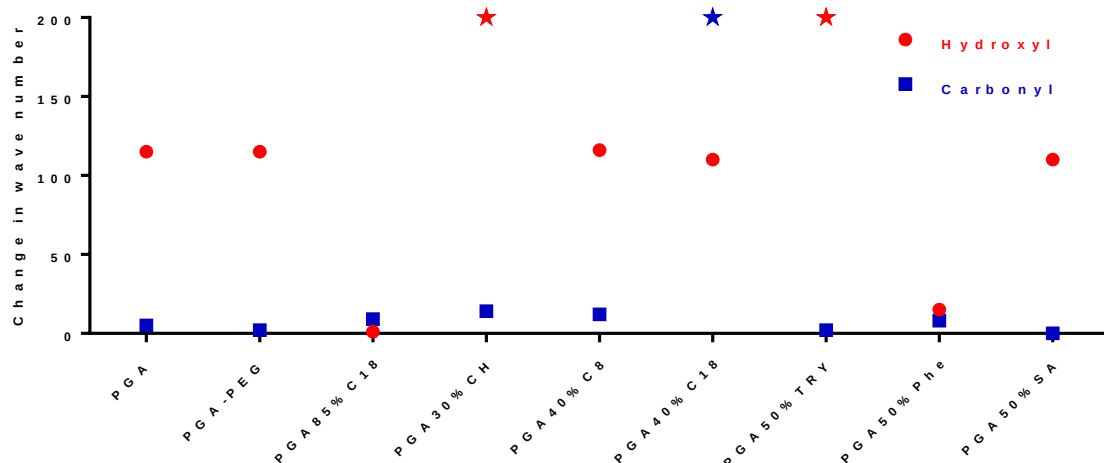


Figure 3-18 A plot of the change in wavenumber (cm^{-1}) of the o-H and carbonyl bond peaks observed in teniposide loaded drug-polymer films. The red and blue stars represent the drug-polymer films spectra with missing O-H and carbonyl peaks respectively.

3.3.3 Contact angle

Contact angle measurement is the second method that was used to try to explain the drug retention percentages obtained in the release experiments. The contact angle measurements were taken to measure the hydrophobicity and wettability of the drug and polymers and the changes that occurred in the drug-polymer films because of interactions between drug and polymer.

The contact angle recorded for etoposide, etoposide phosphate and teniposide were 80.0, 60.3 and 70.0°. The contact angles measured for the polymers, drugs and drug-polymer films are shown in Table 3-1.

Polymer (°)	Etoposide (80.0°)	Etoposide phosphate (60.3°)	Teniposide (70.0°)
PGA (61.6)	71.8	63.1	65.7
PGA-PEG (49.9)	68.2	43.8	79.9
PGA85%C18 (99.0)	46.2	96.0	79.1
PGA30%CH (73.5)	96.6	71.4	99.1
PGA40%C8 (60.5)	62.5	33.6	69.7
PGA40%C18 (82.0)	53.9	56.1	67.3
PGA50%TRY (73.5)	29.2	60.6	65.7
PGA50%Phe (72.8)	68.7	62.8	57.4
PGA50%SA (62.7)	55.7	76.3	75.2

Table 3-1 The measured contact angles of the 40% drug-polymer films, each value being an average of three measurements. In brackets beside each drug and polymer are their respective measured contact angles, each value being an average of three measurements.

Physical mixtures of drug and polymer are expected to have contact angle values that are a reflection of a combination of their separate contact angles (84). In physical mixtures, the contact angles measured are

expected to reflect no reorganisation at the surface of the individual components of the mixture, hence no effect on their interactions with water (84). When interactions or uneven aggregation occurs, deviations from the expected contact angle values may occur. However, on this project a thin film was prepared, where a thorough mixing was carried out. The measured CA values of the films should reflect this, and deviations from this are deduced to be due to interactions.

The films used were made up of 40% drug and 60% polymer. To reflect this different percentage of drug and polymer and their expected contribution to the contact angles of the thin films, the following equation was used:

$$CA \text{ drug polymer film} = 40\% CA \text{ drug} + 60\% CA \text{ polymer}$$

Equation 15

Where CA drug-polymer film is the contact angle of the films, 40% CA drug and 60% CA polymer is the contact angle of drug and polymer respectively. Based on the calculations, the calculated expected contact angles are as shown in Table 3-2.

Polymer (°)	Etoposide (°) (32)	Etoposide phosphate (°) (24.1)	Teniposide (°) (28)
PGA (37.0)	69.0	61.1	65.0
PGA-PEG (30.0)	62.0	54.1	58.0
PGA85%Cl8 (59.4)	91.4	83.5	67.4
PGA30%CH (44.1)	76.1	68.2	72.1

PGA40%C8 (36.3)	68.3	60.4	64.3
PGA40%C18 (49.2)	81.2	73.3	77.2
PGA50%TRY (44.1)	77.1	68.3	72.1
PGA50%Phe (43.7)	75.7	67.8	71.7
PGA50%SA (37.6)	69.6	61.7	65.6

Table 3-2 Table showing the calculated contact angles of the 40% drug-polymer films. The values in brackets beside polymers are the calculated contact angle of the polymers. This was obtained by calculating 60% of the measured contact angles of the polymer. The value in the bracket beside the drug was obtained by calculating 40% of the measured contact angle of the drugs.

The next step is to compare the expected or calculated contact angles of the drug-polymer films with the measured ones. The observed changes varied and are shown in Figure 3-19. The positive values show an increase in contact angle in the measured compared to the calculated while a decrease is represented by the negative values. Overall, PGA films showed the smallest changes in contact angle.

Looking at Figure 3-19, the plot of calculated deviation in contact angle measurements, there were at least small changes observed for all the polymers. However, the biggest changes were observed in PGA85%C18 one of the most hydrophobic polymers and PGA50%TRY a less hydrophobic polymer. PGA30%CH and PGA40%C18 were next in terms of high changes observed, both polymers also vary in terms of hydrophobicity.

Etoposide phosphate also showed at least small changes in all the films. The biggest changes observed PGA40%C8 (intermediate hydrophobicity) followed by PGA40%C18 (very hydrophobic) and an increase was observed

in PGA50%SA (hydrophilic). The results here showed no correlation between contact angle change and hydrophobicity of the polymers.

In all the films, teniposide spectra showed small changes when mixed with polymers. Its highest changes in PGA30%CH and PGA-PEG, some of the most hydrophobic and hydrophilic polymers respectively. PGA40%C18 and PGA50%Phe showed a similar reduction in contact angle while PGA85%C18 and PGA50%SA showed a similar increase.

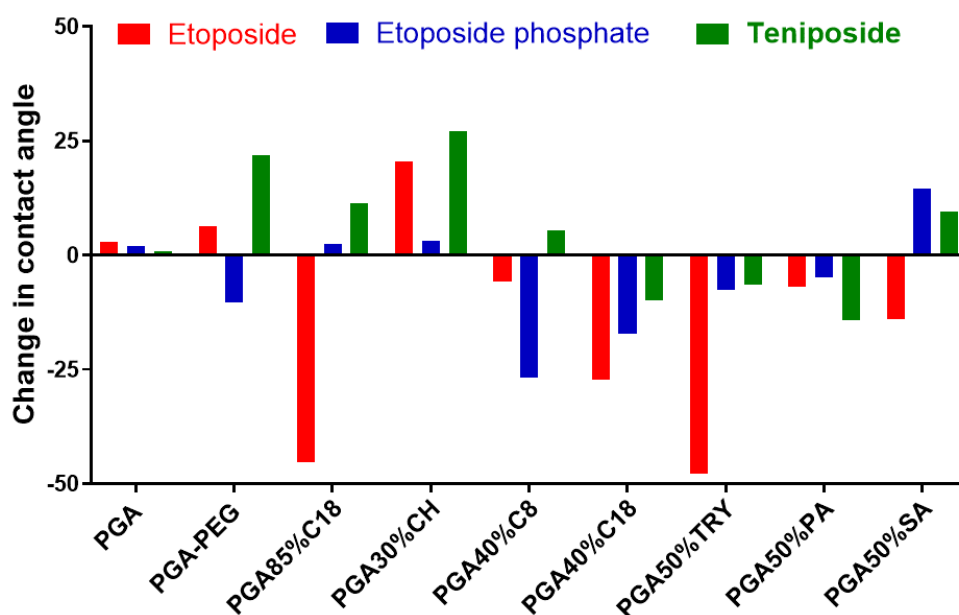


Figure 3-19 A plot of the increase or decrease in contact angle observed in the drug-polymer films relative to expected linear combination of drug polymer contact angles (calculated using equation 15). The negative contact angles show a decrease and the positive values show an increase.

3.4 Discussion

3.4.1 Release experiments

The amount of drug retained is the focus on this project, hence the release results will be expressed in terms of retention rather than drug release as used in the experimental descriptions. The majority of polymers despite having a wide range of hydrophobicity as seen in their estimated logP values (see Table 2-1) have similar retention of etoposide which is low. There was, therefore, no correlation observed between the retention of etoposide being a hydrophobic drug (logP 1.16) and the hydrophobicity of the polymers. Etoposide's highest retention was by PGA50%SA which is very hydrophilic. The next best group made up of PGA, PGA85%C18 and PGA50%TRY having slightly better retention but this group also varied in hydrophobicity.

The retention of etoposide phosphate; the most hydrophilic drug (logP 0.51) by the polymers was very high compared to etoposide and teniposide. The retention was highest in PGA30%CH; one of the most hydrophobic polymers. This is surprising, as it may be expected that the most hydrophilic drugs may be most retained in the most hydrophilic polymers. The next best group of four polymers that retained a high amount of etoposide phosphate have varying hydrophobicity from one of the most hydrophobic (PGA85%C18) to intermediate hydrophobicity and the very hydrophilic ones. The final group with the least retention of etoposide phosphate also have varying hydrophobicity ranging from intermediate to very hydrophilic. For each of these groups of polymers, there was no correlation between the retention of etoposide phosphate and hydrophobicity of the polymers.

Teniposide the most hydrophobic drug (logP 2.78) was least retained by the polymers. The only exception is PGA50%TRY (intermediate hydrophobicity) which retained the most teniposide. The high hydrophobicity of teniposide did not lead to a corresponding high retention by the most hydrophobic polymers. PGA50%Phe which is similar to PGA50%TRY in terms of hydrophobicity showed a different pattern of drug retention.

In the literature, a lot of emphases is placed on the role played by the hydrophobic interactions between drug and polymer on the ability of the polymer to incorporate a drug and retain it (222). Hydrophobic drugs are expected to be retained better because they are less soluble in water but more soluble in the polymer. However, the results obtained from nanoparticle characterisation does not usually show a clear link between hydrophobicity and drug incorporation and retention.

Shah *et al* attempted to demonstrate the link between the extent of polymer substitution and consequently hydrophobicity on the rate of drug release and the amount of burst release demonstrated. They observed a slower release and low burst release of etoposide from their most hydrophobic polymer. They explained that this was due to a slower rate of diffusion of etoposide out of the hydrophobic polymer matrix. The results from this study do not agree with this explanation, the most retention of etoposide was by PGA50%SA the most hydrophilic while etoposide phosphate was most retained by the most hydrophobic polymer. This shows that there is likely to be an alternative explanation for the drug release pattern.

Puri (2010) provided alternative explanations beyond drug-polymer hydrophobicity for the release pattern of drugs from polymers, in her thesis, where the effect of functionalization of PGA on the properties of anti-cancer loaded nanoparticles was studied. A faster release of etoposide

from acylated PGA polymer (PGA_{40% C8} and PGA_{100% C8}) compared to the aminoacyl substituted PGA polymers (5% tryptophan and 5% tyrosine) (99) was reported. She suggested that etoposide retention by the polymers may have been due to the ability of the hydrophobic amino acids to hold the drug in the core. Furthermore, Puri reported a slow release of etoposide phosphate from 100% acylated PGA and the PGA backbone. Etoposide phosphate's lowest retention was by 5% tryptophan conjugated polymer. It was concluded that the different release profile could be due to increased interaction because of the formation of ionic bonds with tryptophan. These results show variation in the release profile of the drugs.

Puri's work, which is the original inspiration for this work suggested other interactions were taking place. This shows the need to use methods that monitor both bond formation and changes in contact angles, to provide scientific evidence for these release behaviours of drugs from these polymers. These are considered in the next sections.

3.4.2 Fourier transform infrared spectroscopy

The FTIR measurements were carried out to determine the type of drug-polymer interactions and the extent due to the shifts in peak position. The IR peaks of the specific functional groups present in the drugs and polymer and the changes to the type of peaks and their positions were noted. The shift of unique bands for each component is an indication of the involvement of the specific functional group in the interactions (223). In this study, the changes in the FTIR spectra of the drug-polymer films were studied and the changes observed in the different peaks varied. The carbonyl peaks, for example, remained in the films' spectra but with varying intensity and sometimes the peak was no longer present at the expected positions.

Shifts in peaks signify interaction and can be further used to determine interaction between drug and polymer (223). The hydrogen bond formed through interactions between drug and polymer would also be expected to lead to changes in the O-H bond FTIR peaks. Hydrogen bonds can be formed between drug and drug, drug and polymer and with the solvents. The formation, however, depends on the functional groups present in the molecules. Looking at the structure of the drugs in Figure 1-12, the oxygen atoms of the carbonyl and hydroxyl are potential proton acceptors while the hydrogen atoms are potential proton donors. In the case of the polymers, the number of free hydroxyl groups varied with the extent of conjugation with the side chain (88). This would also impact the opportunity for hydrogen bonding.

The FTIR spectra obtained for etoposide-polymer films varied across the different polymers. PGA50%TRY's relatively lower release of etoposide and the absence of the OH and C=O in the films is possibly due to extensive changes in bond formation involving these two functional groups. The absence of these peaks indicate the possible formation of hydrogen bonds. This is good evidence that there is good interaction between the drug and polymer which could lead to retardation of the release of the drug.

Etoposide phosphate has potential for ionic interactions via its phosphate functional group (99) in addition to various sites available for hydrogen bond. The OH peak change was highest in PGA, similar to what was observed with etoposide. The carbonyl peak was no longer present in most of the etoposide phosphate incorporated films (except PGA50%TRY). Etoposide phosphate was the most retained drug overall, perhaps the missing peaks play a role in explaining this. In the case of teniposide, the highest changes in wavenumber occurred in PGA50%TRY which had the best retention of teniposide in 24 hours and gave a spectrum with a missing OH peak.

A missing peak can also be interpreted as evidence of good interaction between drug and polymer which could improve the polymer's ability to retain a drug. Zheng *et al* postulated in their work involving the preparation of solid dispersions of nimodipine with HPMC, polyvinyl pyrrolidone/vinyl acetate copolymer (PVP/VA, Plasdone S630), and ethyl acrylate, methyl methacrylate polymer that the N-H stretching peak not being present in the FTIR spectrum of the dispersion was a strong proof of the interactions between nimodipine and the polymers (224). This may provide explanations especially for the results with etoposide phosphate and teniposide with PGA50%TRY.

In addition to this, the effect of hydrogen bonding on the dissolution profile of solid dispersions was demonstrated in Karavas's study (225) of the mechanism of release of felodipine from poly(vinyl pyrrolidone) nanodispersions, changes in the dissolution of the sparingly soluble felodipine were observed. It was reported that the changes to the dissolution mechanism of the solid dispersions were due to the presence of the hydrogen bonding interactions between the drug and polymer. The interactions were shown by the shifts in the N-H bond stretching of felodipine after interacting with oxygen in the polymers. They further stated that the hydrophilicity of the polymer was not responsible for the changes observed. In addition, Gubskaya *et al* proposed a possible contribution of polymer-polymer hydrogen bond interactions on drug release from a polymer matrix (221). They used Molecular dynamics (MD) simulations to monitor hydrogen bond interactions and observed a faster release of Voclosporin from the drug-polymer complexes which showed a higher number of polymer-polymer interactions. This shows that in observing the interactions between the drug and polymer, a further step

to be added is the type of interactions present and their possible impact on drug release or incorporation into the polymers.

Our results so far indicate that the interaction between drug and polymer is dependent on other factors beyond their hydrophobicity or hydrophilicity. It also shows that hydrogen bonding may play a part in the release behaviour of drugs from the films. However, the highest shift in peak numbers did not correspond to the lowest drug release in 24 hours (see Table 3-3, Table 3-4 and Table 3-5). The implication of this is that the FTIR data does not fully explain the drug release obtained from the films and further explanations are still required.

3.4.3 Contact angle

Looking at the results obtained in this study for etoposide (CA; 80.0°, LogP; 1.14), the contact angle obtained for etoposide-PGA85%C18 film (PGA85%C18: CA; 99.0°, predicted logP; 6.06) and etoposide-PGA50%TRY (PGA50%TRY: CA; 73.5°, predicted logP; 1.12) were much lower than expected. An explanation for this is that within the polymer matrix, there are domains of groups of molecules associated together where the more hydrophilic functional groups – hydroxyl, succinate are now at the surface.

For etoposide phosphate (CA; 60.3°, logP; 0.51), Etoposide phosphate-PGA30%CH (PGA30%CH: CA; 73.5°, logP; 6.06) gave the highest contact angle increases while etoposide phosphate-PGA40%C8 (PGA40%C8: CA; 60.5°, logP; 2.27) led to the highest reduction. In the former case, it may mean that the hydrophobic functional groups such as the acyl, alkyl and cholesterol groups are prominent at the surface, while in the latter the hydrophobic groups may become more buried within the domain in the polymer matrix. To compare this with the release results, the PGA30%CH retained the most amount of etoposide phosphate, an unexpected result from the most hydrophilic drug and one of the most hydrophobic

polymers. The FTIR spectra of the etoposide phosphate containing PGA₃₀%CH and PGA₄₀%C8 films show peak shifts in the OH and disappearance of the carbonyl peaks. The high retention of etoposide phosphate may have been due to the longer wetting time combined with the intra and inter molecular interactions.

The contact angle of Teniposide-PGA₃₀%CH (Teniposide CA; 70, logP; 2.78) showed the greatest increase, followed by PGA-PEG (CA; 49.9, logP; 0.71). The unexpected result here is mainly for PGA-PEG because of its hydrophilicity. While peak shifts were observed in the films' spectra, these did not correlate with good results in this case.

The ability of the dissolution media to penetrate the polymer matrix is important in the dissolution process. This penetration depends on wettability which can be measured using contact angle. This property reflects the hydrophobicity of the drug and polymer at the surface of the materials (226). To study the changes in the contact angle of drug and polymer upon mixing, contact angle measurements were taken. Hydrogen bonds, ionic bonds and dipole-dipole bonds can be formed between drug and polymer, drug and solvent. Drug-polymer interactions may change the wettability of polymers due to changes in the mobility of the polymer chain and cohesion between the chains (84). Also, when intermolecular bonds are formed between drug and polymer there is a possible alteration of the coordination of the chains present in the polymer (220).

Assuming that there is no interaction between drug and polymers, a contact angle reflecting the properties of the combination of the two individual components is expected (84). However, when interactions occur, their interaction with water is changed because the hydrogen bonds and side groups get rearranged. This leads to a change in the interaction of the resulting drug-polymer mixtures with water leading to unexpected changes in drug polymer contact angles higher or lower than the expected.

There are expected contributions based on the extent of hydrophobicity of the polymers and drugs. Redistribution of the side groups that are exposed at the solid air interface also occurs. All of these can influence the wettability of the substances involved. The large changes in contact angles shows that the functional groups now on the surface upon rearrangement due to interactions are now more hydrophilic or hydrophobic (84).

3.4.4 Summary

Table 3-3, Table 3-4, and Table 3-5 show summaries of all the drug-polymer screening results. A lot of emphases is placed on high drug retention in this project because the intended drug delivery system is required to hold the drug long enough for uptake into tumours where drug action is required. The overall goal on this project is to ensure that the drug retained nanoparticles at the point of uptake into tumours are high and there is minimal drug loss due to burst release before tumoural uptake.

Polymer	log P of repetitive units	Contact angle	Change in contact angle	% drug retained	FTIR; Change in wavenumber	
					OH	C=O
PGA-PEG	0.71	49.9	6.2	28.5	-126	14
PGA-40%C8	2.27	60.5	-5.8	26.7	-101	19
PGA	0.96	61.6	2.8	38.0	-155	-15
PGA50%SA	-0.03	62.7	-13.9	49.2	44	8
PGA50%Phe	1.06	72.8	-7	31.6	-49	21
PGA50%TRY	1.12	73.5	-47.9	35.5		
PGA30%CH	6.06	73.5	20.5	17.7		24
PGA40%C18	5.45	82	-27.3	22.7	79	16
PGA-85%C18	6.06	99	-45.2	35.8	-37	16

Table 3-3 Summary of the drug polymer screening results obtained for etoposide showing the log P of the repetitive units and contact angles of the polymers, the changes in contact angles and wavenumbers and the percentage drug released in 24 hours in the etoposide loaded drug polymer films. The red highlighted line represents the polymer-drug combination chosen for further study.

Polymer	log P of repetitive units	Contact angle	Change in contact angle	% drug retained	FTIR; Change in wavenumber	
					OH	C=O
PGA-PEG	0.71	49.9	-10.3	76.8	-12	
PGA-40%C8	2.27	60.5	-26.8	58.2	71	
PGA	0.96	61.6	2	39	181	
PGA50%SA	-0.03	62.7	14.6	71		
PGA50%Phe	1.06	72.8	-5	82	43	
PGA50%TRY	1.12	73.5	-7.7	28		-4
PGA30%CH	6.06	73.5	3.2	96.1	50	
PGA40%C18	5.45	82	-17.2	45	135	
PGA-85%C18	6.06	99	2.5	76.2	70	

Table 3-4 Summary of the drug-polymer screening results obtained for etoposide phosphate showing the log P of the repetitive units and contact angles of the polymers, the changes in contact angles and wavenumbers and the percentage drug retention in the etoposide phosphate loaded drug-polymer films. The red highlighted line represents the polymer-drug combination chosen for further study.

Polymer	log P of repetitive units	Contact angle	Change in contact angle	% drug retained	FTIR; Change in wavenumber	
					OH	C=O
PGA-PEG	0.71	49.9	21.9	18.7	115	2
PGA-40%C8	2.27	60.5	5.4	9.6	116	12
PGA	0.96	61.6	0.7	19.4	115	5
PGA50%SA	-0.03	62.7	9.6	15.3	110	0
PGA50%Phe	1.06	72.8	-14.3	25.4	-15	8
PGA50%TRY	1.12	73.5	-6.4	71		2
PGA30%CH	6.06	73.5	27	11.3		-14
PGA40%C18	5.45	82	-9.9	0.4	110	
PGA-85%C18	6.06	99	11.4	12.3	-1	9

Table 3-5 Summary of the drug polymer screening results obtained for teniposide showing the log P of the repetitive units and contact angles of the polymers, the changes in contact angles and wavenumbers and the percentage drug retention in the etoposide phosphate loaded drug-polymer films. The red highlighted line represents the polymer-drug combination chosen for further study.

In choosing polymer drug combinations for further study, there were instances of high changes in contact angle and high changes in wave number which do not equate to changes in retention. While a full explanation of the reasons behind the changing retention may not yet be possible, the polymers choices were based on the high retention accompanied by highest wavenumber and high contact angle changes where applicable.

Based on the results, PGA85%C18 was picked for etoposide because though the release is second lowest, it has a higher contact angle change and higher combined change in wavenumber compared to PGA50%SA. PGA85%C18 was picked for etoposide phosphate because it had a much higher change in wavenumber compared to PGA30%CH and PGA50%Phe which had the most retention. For teniposide, PGA50%TRY is the polymer that retained by far the most teniposide in 24 hours with some change in contact angle and wavenumber.

3.5 Conclusion

In this chapter, the interactions between etoposide, etoposide phosphate and teniposide and nine polymers PGA, PGA-PEG, PGA40%C8, PGA40%C18, PGA85%C18, PGA30%CH, PGA50%TRY, PGA50%Phe and PGA50%SA were assessed using three different approaches; release experiments, FTIR spectroscopy and contact angle measurements. The FTIR and contact angles measurements were explored as an attempt to find an explanation for the release results obtained. Multiple approaches are required to conclusively show drugs and polymers interact (83).

The peak shifts and side-chain rearrangement have been shown to play a possible role in the drug release behaviour of the polymers. However, a direct proportionality between drug release and contact angle change and FTIR peak shifts was not demonstrable, suggesting that these factors only account for part of the full explanation.

The next chapter is focussed on preparing nanoparticles from the matched drugs and polymers (Table 3-6). To investigate the properties of the nanoparticles, and to see if the results from the drug-polymer screening were reflected in the nanoparticles where more complex factors may be present.

Drug	Polymer
Etoposide	PGA85%C18
Etoposide phosphate	PGA85%C18
Teniposide	PGA50%TRY

Table 3-6 The selected polymers for each drug based on results from release experiments, changes in contact angle and wavenumber

Chapter 4

4 Preparation and Characterization of nanoparticles

4.1 Introduction

The focus of this chapter is to prepare and characterize the nanoparticles from the drug-polymer choices made. To achieve the requirement for successful drug delivery of adequate dose at the target site in nanomedicine, nanoparticles must be carrying enough drug. The drug: carrier ratio must be controlled to ensure that sufficient drug can be delivered to the target site and that the resulting polymer concentration at the target site is not too high to cause toxicity (78). In this project, a lot of emphasis was laid on the importance of the drug to be retained within the nanoparticles until cellular uptake occurs. It was also important to ensure that nanoparticles that are taken up into the cells are carrying high drug content.

The entry of nanoparticle into the cell is by endocytosis, which involves engulfment of the cargo in membrane invaginations and the formation of endosomes which then deliver the cargo to their various destinations (227). The rate of endocytosis is mainly affected by factors such as particle size, charge and shape of the nanoparticles (228). Other factors that affect endocytosis are cell type, which includes the type and origin of the cell, the endocytosis pathways present in the cells (227). The fate of the particle after endocytosis is not fully understood, however various suggestions as to what happens to nanoparticles have been made. These include entry into lysosomes, accumulation at their intracellular target and recycling out of the cells in form of exocytosis (229). All of these affect the availability of the drug at the target site.

The lysosomal compartment is the expected site of drug release for the nanoparticles on this project, expected to occur after polymer degradation. Degradation may occur due to the pH of the lysosome and also due to the presence of the enzymes such as acid hydrolases, proteases, nucleases, esterases, and lipases. The drug may then be required to move out of the lysosome into the cytosol or nucleus for action. However, it is important to consider the rate, time and mechanism with which this occurs. The intracellular transport of the drug is dependent on the physicochemical properties of the drug as they affect their ability to cross the lysosomal membrane.

Hypothetically, it is expected that the endocytotic process of nanoparticle uptake is saturable. The goal is to avoid saturation of the process with nanoparticles carrying a very low amount of drug and overloading the cells with the excess carrier. The aim is to ensure that the nanoparticles taken up carry a 'high enough' amount of drug. While ensuring adequate drug loading, it is also important that premature drug loss due to burst release and early diffusion out of the nanoparticle is reduced. This is to ensure that the drugs are retained long enough to reach target site.

The technique used in this chapter to prepare nanoparticles is nanoprecipitation, a quick, easy, one-step procedure that has been used to successfully prepare polymeric nanoparticles (104). It involves the precipitation of a polymer as a water-miscible solvent is displaced into the external phase as it comes in contact with the aqueous phase (102). A significant advantage of the technique is the ability to yield small sized particles with narrow distribution (109,230). In addition, the nanoprecipitation method has been used for the successful encapsulation of hydrophilic (104,109) and hydrophobic drugs (117).

The specific aims of these chapter are:

1. To prepare and characterise nanoparticles from the etoposide, etoposide phosphate, teniposide and their chosen polymers.
2. To perform cytotoxicity experiments using MTT assay to compare the cytotoxicity of nanoparticles and free drugs.

4.2 Materials and method

4.2.1 Nanoprecipitation

Etoposide, Etoposide phosphate and teniposide nanoparticles were prepared using an interfacial deposition method as described in section 2.2.2.1.

4.2.2 Separation of unincorporated drug from nanoparticles

The unincorporated drugs in all instances were removed following the method as described in section 2.2.2.2.

4.2.3 Particle size and Zeta potential

The particle size and surface charge of the nanoparticles were determined using the methods described in section 2.2.2.3.

4.2.4 Determination of drug loading

The amount of drug incorporated into the nanoparticles was determined following the method described in section 2.2.2.4.

4.2.5 Determination of Drug Release

The amount of drug released from the nanoparticles was determined using the methods detailed in section 2.2.2.5.

4.2.6 Cytotoxicity assay

4.2.6.1 Determination of optimal numbers of cells per well

The number of cells to be cultured per well was determined as described in section 2.2.2.2.

4.2.6.2 3- (4,5-Dimethyl thiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) Assay

The cytotoxicity of the nanoparticles and free drugs were assessed using MTT assay as detailed in section 2.2.2.3.

4.2.7 Data analysis

The experiments were carried out at least three times except when stated otherwise and results of were analysed using GraphPad prism 7.0. The concentrations of drug in the samples were calculated and used to plot a graph of cumulative release. The release of the nanoparticles was fit into mathematical models to further assess the release profile. Statistical analysis was carried out using excel and GraphPad prism 7.0.

4.3 Results

4.3.1 Nanoprecipitation and Separation of unincorporated drug from nanoparticles

The nanoparticles were prepared by nanoprecipitation and the free drug was removed using low pressure gel permeation chromatography. A proper clean-up process was vital to avoid exaggerated drug loading results and the presence of the free drug which would act in a different manner to the formulation. The peaks eluted for the nanoparticles were completely separated from the drug peaks as judged by the baseline separation of peaks on the elution profile (see Figure 2-10).

4.3.2 Particle size and Zeta potential

The size and charge were determined using dynamic light scattering. The expected particle size range would be below 200 nm, this particle size will increase the potential for efficient cellular uptake and intra-tumoural transport. Table 4-1 shows a summary of the size and charge obtained for all nanoparticle preparations.

Drug	Polymer	NP Particle size diameter $\pm$ SD nm	Zeta potential $\pm$ SD mV
Etoposide	PGA85%C18	191.0 $\pm$ 2.9	-64.7 $\pm$ 1.2
Etoposide phosphate	PGA85%C18	214.0 $\pm$ 19.5	-45.4 $\pm$ 2.4
Teniposide	PGA50%TRY	112.0 $\pm$ 3.0	-48.4 $\pm$ 4.6

Table 4-1 particle size and zeta potential of etoposide, etoposide phosphate and teniposide loaded nanoparticles with the matched polymers PGA85%C18 and PGA50%TRY. The properties were determined using DLS. The results are a mean of three separate experiments $\pm$ standard deviation (SD).

4.3.3 Drug loading

To determine the amount of etoposide and teniposide loaded into the nanoparticles, they were dissolved in 50:50 DCM: DMSO and fluorescence spectrophotometry was used for the determination. HPLC which was the method of determination used earlier for these drugs could not be used because the solvents used to dissolve the polymer (DCM and DMSO) was not compatible with HPLC systems available.

The drug loading obtained for etoposide-PGA85%C18 formulation was $5.0\% \pm 1.0$, $3.5\% \pm 1.0$ was obtained for etoposide phosphate-PGA85%C18 formulation and teniposide-PGA50%TRY showed a drug loading of $13.4\% \pm 2.8$ (Figure 4-1).

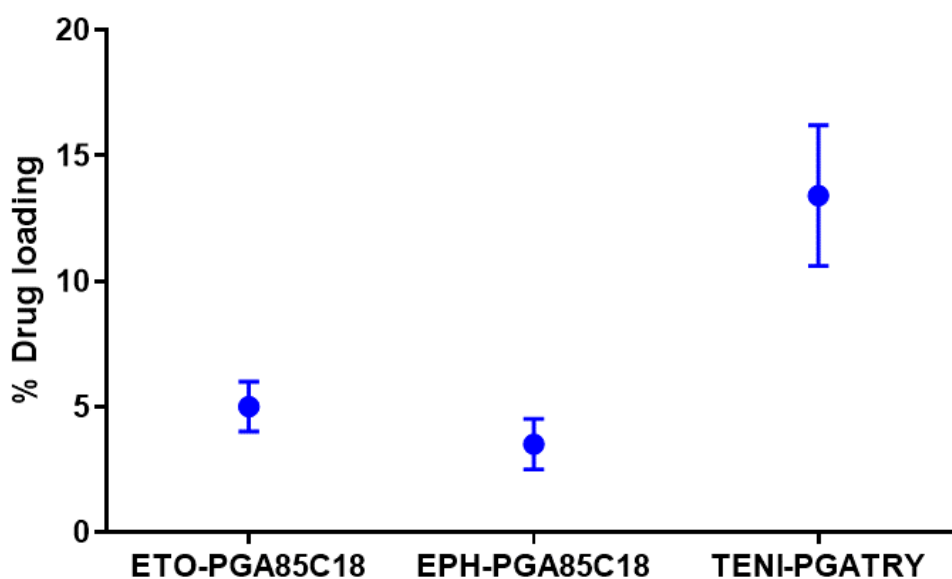


Figure 4-1 A representation of the % drug loading obtained from etoposide-PGA85%C18, etoposide phosphate-PGA85%C18 and teniposide-PGA50%TRY nanoparticles. Error bars correspond to the standard deviation of three separate experiments.

4.3.4 Drug release

Drug release experiments typically show that many nanoparticles have two main problems, a high burst release and a continued rapid release of drug leading to low amounts of drug still encapsulated by the time they reach their target.

The drug release experiments for the nanoparticles on these project was carried out using dialysis and the frozen and thawed samples were analysed using the validated HPLC method described in section 2.2.1.1.2 for etoposide and teniposide and UV spectroscopy (section 2.2.1.1.4) for etoposide phosphate. Nanoparticle suspension samples were placed in the dialysis tube while maintaining sink conditions.

Figure 4-2 shows the release profile of etoposide. The free drug diffused rapidly (green square) into the dissolution media. 50% diffused out in 4 hours and the rest of the drug diffused out within 24 hours. From etoposide loaded PGA85%C18 nanoparticles (red circle), there was a 12% burst release in 4 hours, the cumulative release rate slowed down from 4 hours with 22% and up to 34% released after 24 and 48 hours respectively. The etoposide spiked empty PGA85%C18 nanoparticles (blue triangle) has a release rate similar to the etoposide loaded nanoparticles. In 4 hours 24% was released and the release was sustained from then till 24 hours where 27% was released and 35% released after 48 hours.

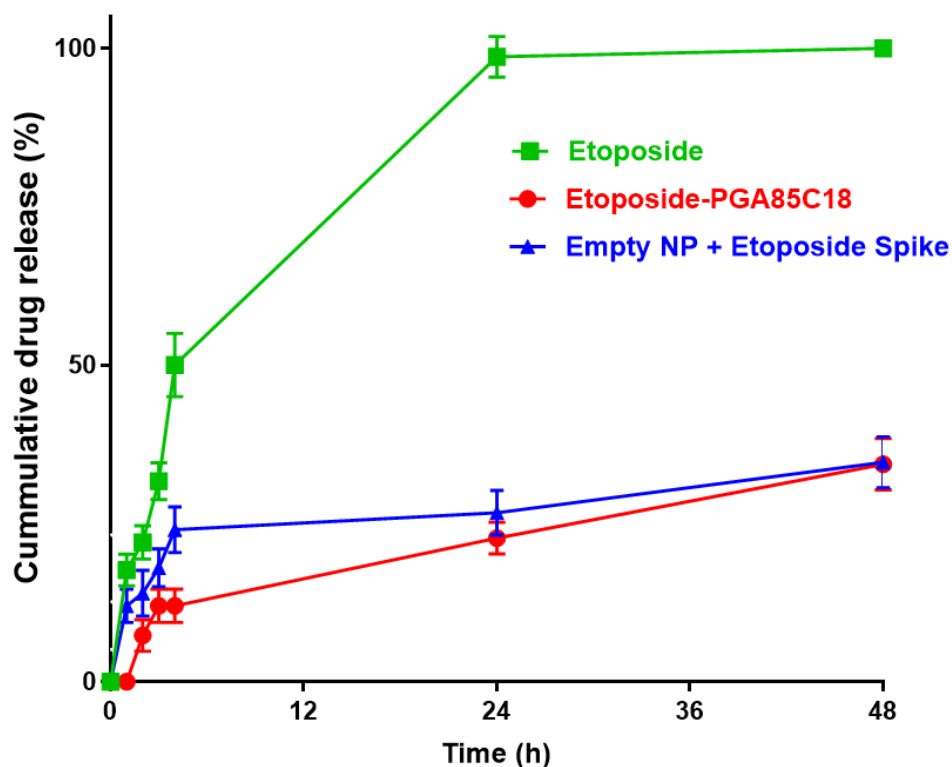


Figure 4-2 Release from etoposide loaded nanoparticles with PGA85%C18 determined by dialysis showing free drug release and release from etoposide spiked empty nanoparticles.

Figure 4-3 shows the release profiles of etoposide phosphate from PGA85%C18 nanoparticles (red circle), free etoposide phosphate (green square) and etoposide phosphate from empty PGA85%C18 nanoparticles spiked with etoposide phosphate (blue triangle).

With the free etoposide phosphate, 50% diffused rapidly into the dialysate in 4 hours and the remaining diffused out within 24 hours. From the PGA85%C18 nanoparticles (red circle), there was a very low burst release of 10% etoposide phosphate in 4 hours, with a cumulative release of 16% and 20% released after 24 and 48 hours respectively. Etoposide phosphate spiked PGA85%C18 also showed initial burst release with 34% etoposide phosphate released in 4 hours, then 72% after 24 hours and the rest was released in 48 hours.

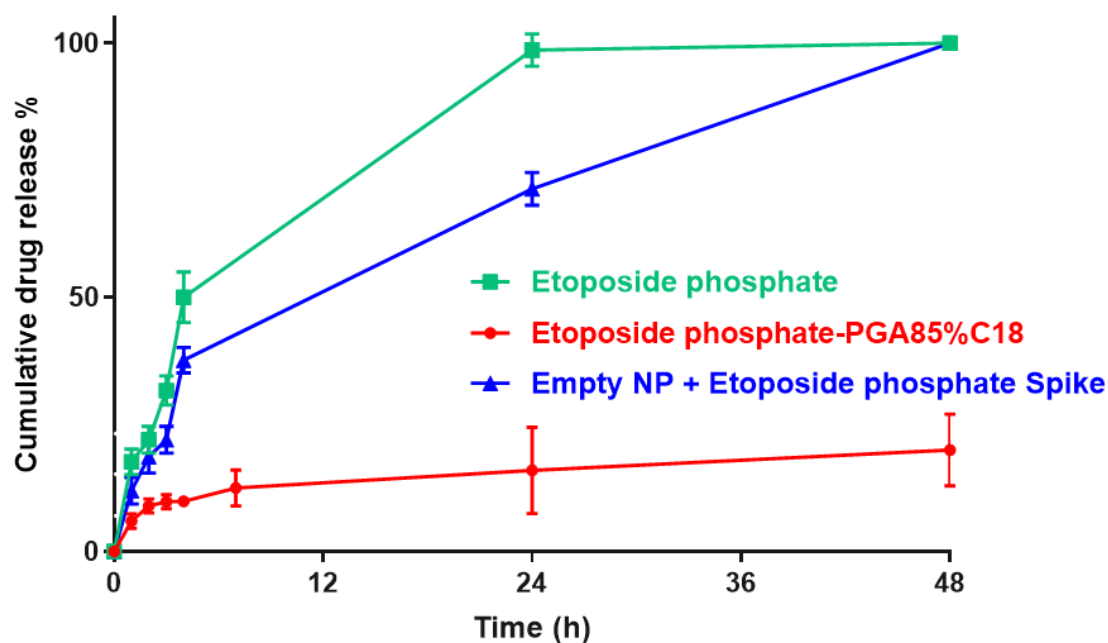


Figure 4-3 Release from etoposide phosphate loaded nanoparticles with PGA85%C18 determined by dialysis showing free drug release and release from etoposide phosphate spiked empty nanoparticles.

The release profile of teniposide is depicted by Figure 4-4. Free teniposide diffused rapidly into the dialysate; about 50% in the first 4 hours, while the rest diffused out within 24 hours. A burst release of about 27% occurred in the first 4 hours for teniposide loaded PGA50%TRY nanoparticles (red circle). The release slowed down with a cumulative release of only about 35% released after 24 hours and 48% released after 48 hours. For teniposide spiked empty PGA50%TRY nanoparticle, an initial burst release of about 50% was observed in the first 4 hours. The release then slowed down and 60% cumulative release was observed at the 24 hours mark and 83% at the 48 hours mark.

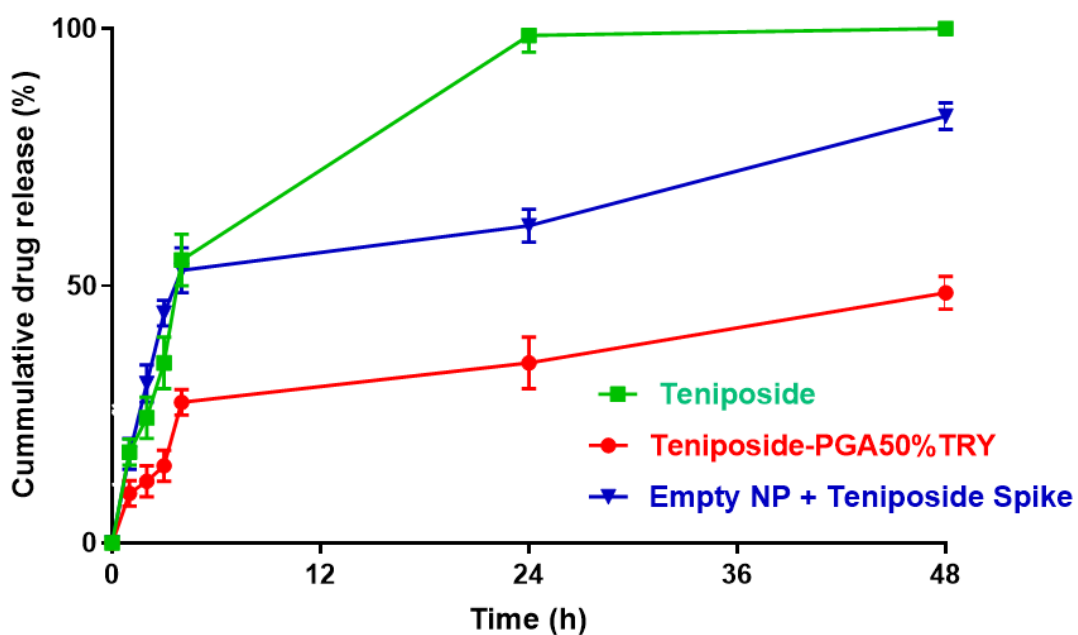


Figure 4-4 Release from Teniposide loaded nanoparticles with PGA50%TRY determined by dialysis showing free drug release and release from teniposide spiked empty nanoparticles.

System	Burst release (drug release in 4 hours)	Drug released in 24 hours	Drug released in 48 hours
Etoposide-PGA85%C18	12%	22%	34%
Etoposide phosphate-PGA85%C18	10%	16%	20%
Teniposide - PGA50%TRY	27%	35%	48%

Table 4-2 Summary of the drug release results of the drug loaded polymer nanoparticles; showing the burst release in 4 hours and the cumulative drug release in 24 and 48 hours.

4.3.4.1 Drug release modelling

Fitting of various mathematical models is done to further understand the drug release behaviours of the formulations. The zero order, Higuchi (Figure 4-5), first order (Figure 4-6) and Korsmeyer Peppas (Figure 4-7) models are used in this project. These models were chosen because they are all applicable to different matrix systems and could provide a possible explanation for the mechanism of release of the DLPNP.

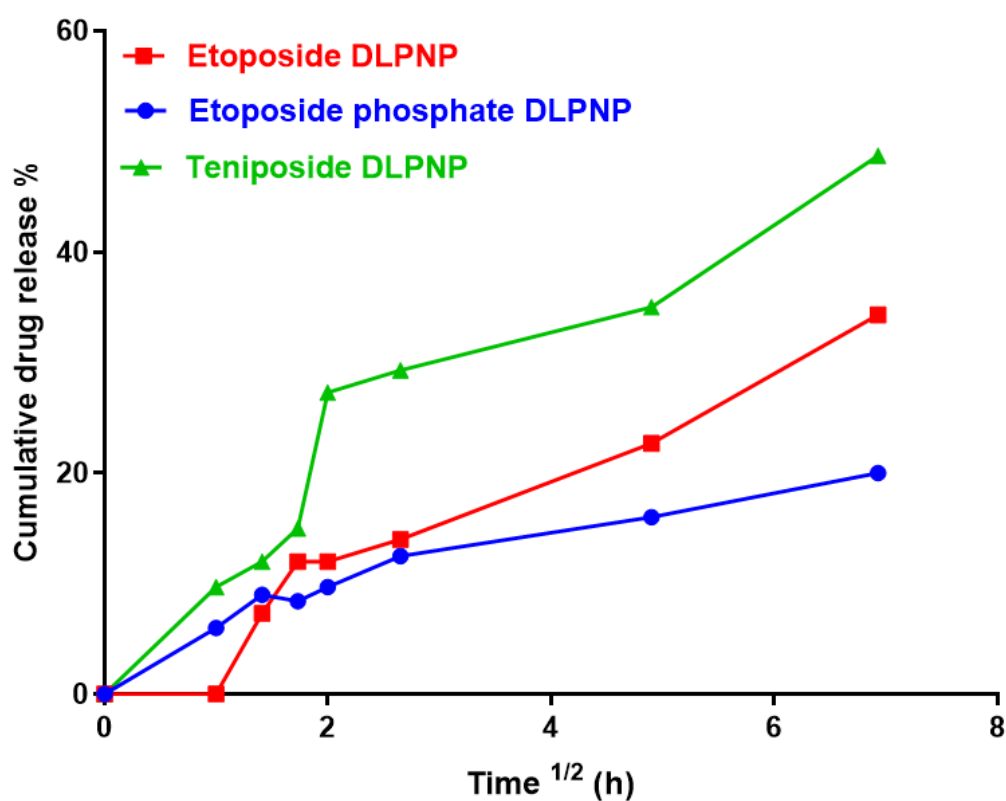


Figure 4-5 Higuchi plots for Etoposide DLPNP, Etoposide phosphate DLPNP and teniposide DLPNP.

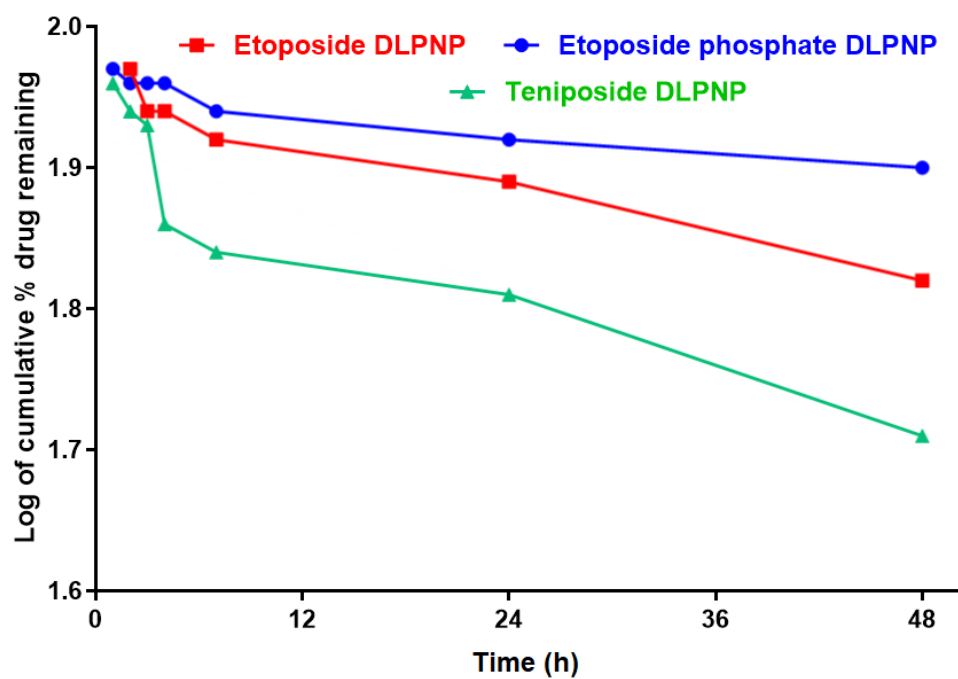


Figure 4-6 First order plots for Etoposide DLPNP, Etoposide phosphate DLPNP and teniposide DLPNP.

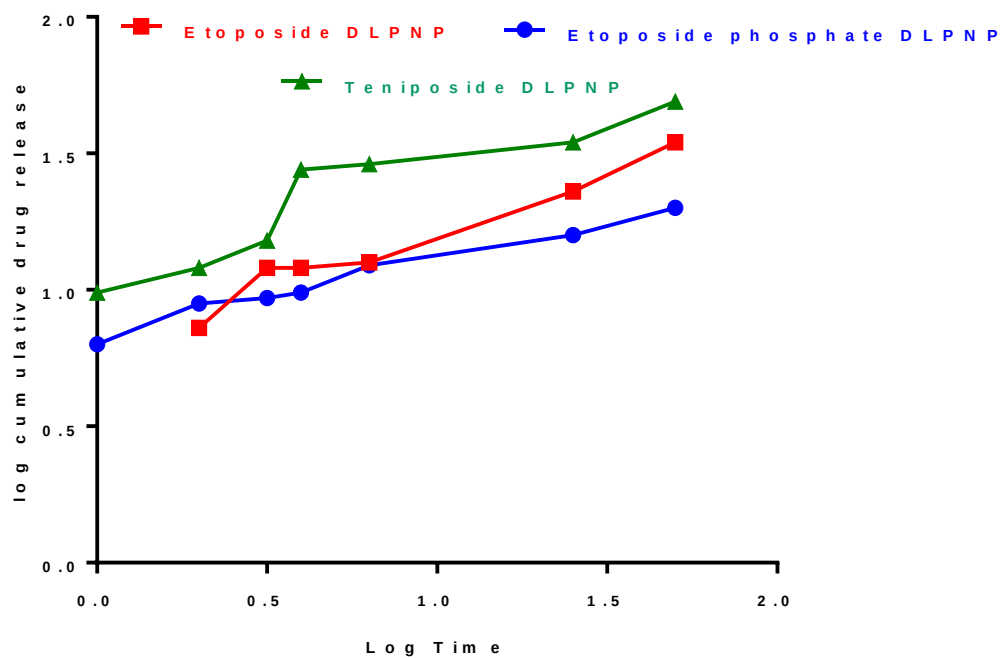


Figure 4-7 Korsmeyer Peppas model plots for Etoposide DLPNP, Etoposide phosphate DLPNP and teniposide DLPNP.

Model	Etoposide DLPNP	Etoposide phosphate DLPNP	Teniposide DLPNP
Zero order	0.8798	0.777	0.8116
First order	0.968	0.9111	0.903
Higuchi	0.953	0.9099	0.9269
Korsmeyer Peppas	0.9604	0.9762	0.8958

Table 4-3 Table of the R square value of the linear regression of the plots of various drug release models of all the drug loaded polymer nanoparticles. First order, Korsmeyer Peppas and Higuchi appear to be the highest for etoposide DLPNP, etoposide phosphate DLPNP and teniposide DLPNP respectively.

System	n value
Etoposide DLPNP	0.4209
Etoposide phosphate DLPNP	0.3035
Teniposide DLPNP	0.5018

Table 4-4 The Korsmeyer Peppas exponent (n) values obtained for the DLPNP prepared.

None of the curves shows a good linearity which would be expected of a diffusion mechanism corresponding well to the drug release models. The highest degree of correlation indicates that the mathematical model that correlates best with the release kinetics of the formulation. The first order

and Korsmeyer Peppas were the highest for etoposide DLPNP, the Korsmeyer Peppas was the highest for etoposide phosphate DLPNP while the Higuchi was the highest for Teniposide DLPNP Table 4-3. Furthermore, the Korsmeyer exponent values (n values) for etoposide and etoposide phosphate DLPNP are all below 0.45 while the teniposide DLPNP n value is 0.5018 Table 4-4. The n value is used to determine the what drives the release of the drugs as described in section 1.4.5.2.2.

4.3.5 Cell culture

The absorbance measured per well reflects the amount of formazan formed from the reaction of the metabolically active cells with the MTT reagent. Optimising the cells ensures that the cell number used for the cytotoxicity experiment is within the linear range. As seen in Figure 2-14, a linear relationship was obtained between the cell number and absorbance from 0 – 10000 cells/well.

After the optimization of cell number, cytotoxicity experiments (MTT) were then carried out on the nanoparticles and free drug. The cytotoxicity after 24 hours was evaluated in UW228-3 MB cell lines and compared with free drugs. The cell line and culture conditions were chosen so that the results were comparable with those obtained by Ivanov (117), however, an MTT assay was used in the present work rather than the methods spheroid volume, resazurin metabolism and acid phosphatase activity used in that project. The aim of the cytotoxicity experiment is mainly to see the differences in the cytotoxic effects of the DLPNPs compared to the free drugs.

As shown in Figure 4-8, Figure 4-9 and Figure 4-10, the cytotoxic activity of the nanoparticles increased as dose increased. The dose response curves of etoposide PGA85% C_{18} starts to show some cell death at a lower concentration compared to the free etoposide. While the reverse is the

case for etoposide phosphate PGA85%C18 and Teniposide PGA50%TRY. The free etoposide phosphate and Teniposide curves showed less steep slopes compared to their respective DLPNP. The dose response curve for etoposide phosphate PGA85%C18 showed its first sign of response at a higher concentration compared to the free drug. A steep slope then began to form which does not reach a 50% reduction in viability by the dose used. The IC₅₀ for this particular curve was extrapolated using graph pad prism to enable us to determine a figure for the IC₅₀ for ready comparison. Teniposide PGA50%TRY also showed a later response but shows a steeper slope and more cell death at higher concentrations. All the curves, however, seemed to show some cell viability at high concentration. The half maximal inhibitory concentrations (IC₅₀) of the nanoparticles and drugs were determined from the dose response curves and shown in Table 4-5.

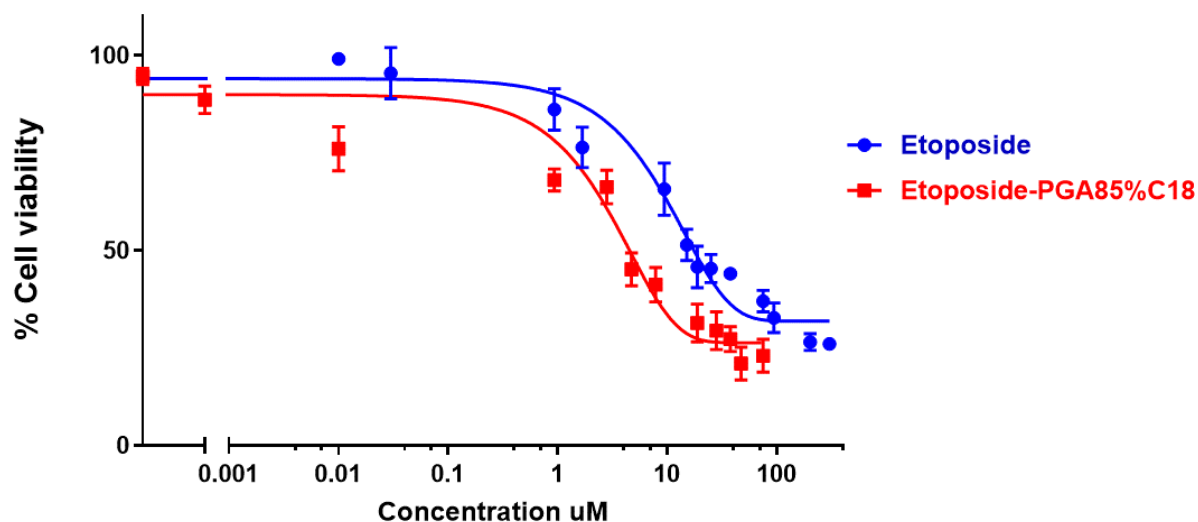


Figure 4-8 Dose response curve obtained from MTT assay conducted to assess the cytotoxicity of free etoposide and etoposide-PGA85%C18

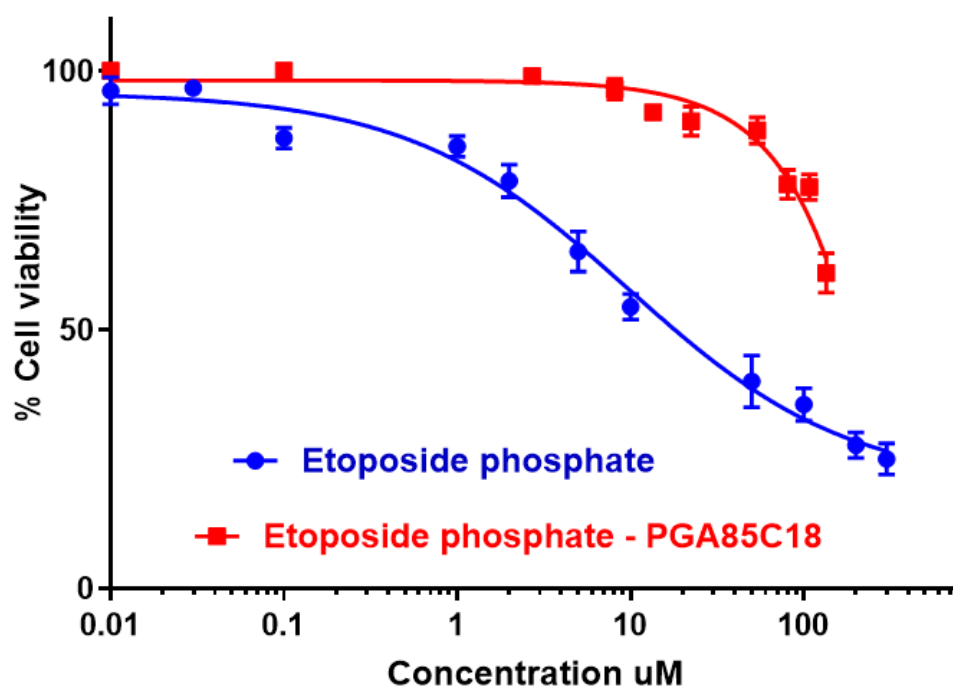


Figure 4-9 Dose response curve obtained from MTT assay conducted to assess the cytotoxicity of free etoposide phosphate and etoposide phosphate-PGA85% C_{18}

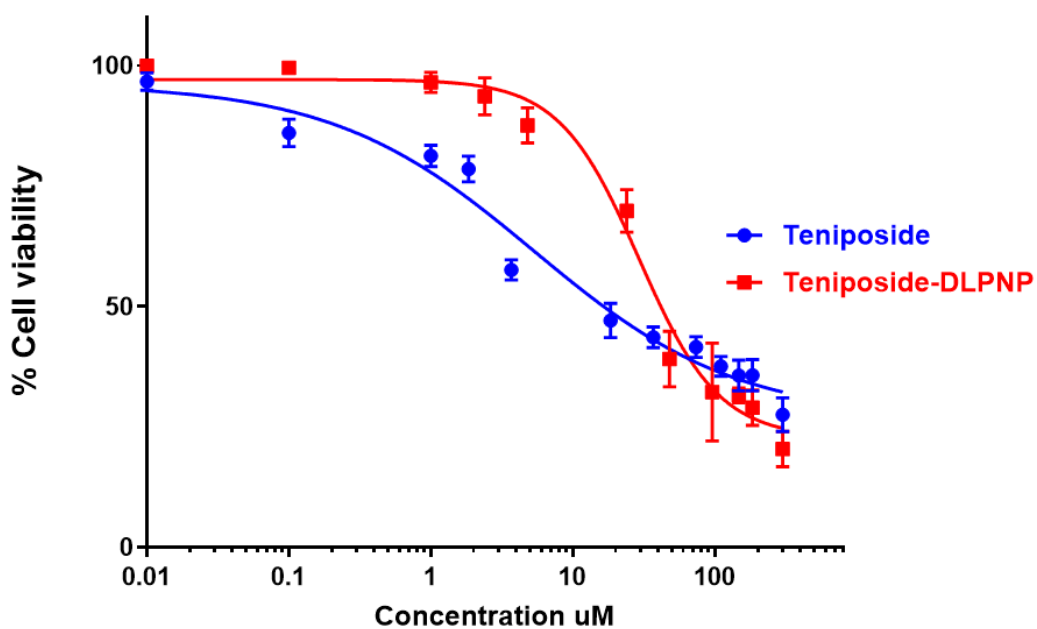


Figure 4-10 Dose response curve obtained from MTT assay conducted to assess the cytotoxicity of free teniposide and teniposide-PGA50% TRY .

System	IC ₅₀ μ M
Etoposide (free drug)	20.5
Etoposide-PGA85%C18	5.0
Etoposide phosphate (free drug)	18.0
Etoposide phosphate -PGA85%C18	174.0
Teniposide (free drug)	16.0
Teniposide-PGA50%TRY	41.6

Table 4-5 The IC₅₀ drugs and nanoparticles obtained from the dose response curves plotted from the MTT cytotoxicity experiments.

4.4 Discussion

4.4.1 Particle size and zeta potential

The particle size and surface charge are important parameters that can impact the pharmacokinetics and biodistribution of the nanoparticle (150). Some of the factors that have been shown to affect the particle size of nanoparticles include polymer molecular weight (231), the method of formulation and formulation parameters (232). The nanoparticles prepared in this project are intended for local delivery into the brain from where uptake into MB cells will occur. This uptake of the nanoparticles into the tumour depends on the physicochemical properties of the nanoparticle (233).

The role of the particle size to the successful delivery of nanoparticles to tumours was explained in Wilhelm *et al* review of the delivery of nanoparticles to tumours. They observed that formulations with particle size diameter below 100 nm showed a slightly higher delivery than larger nanoparticles (154). The most important for us on this project is cellular uptake and despite the requirement of particle size below 100 nm for efficient tumour delivery, some studies observed cellular uptake with nanoparticles above 200 nm. For example, Teniposide loaded albumin nanoparticles with particle size of about 200 nm have been shown to have a higher time-dependent cellular uptake by C6 glioma cell lines, than free teniposide (234). Also, the particle size of the Rhodamine B Isothiocyanate (RBITC) labelled PGA nanoparticles prepared by Meng *et al* was 176 nm and they were taken into the endosomes and lysosomes of DAOY MB cell line (96). There was also preferential uptake into the MB cell lines compared to normal cells. Nanoparticles with particle sizes ranging from 25 nm to 200 nm have been reported to be taken up into tumour cells. This shows that while the delivery efficiency is higher in particle size below 100 nm, cellular uptake can be obtained for nanoparticles 200nm and below.

The nanoparticles prepared in this project were all below 200 nm apart from etoposide phosphate-PGA85%C18, which gives them a possible chance of uptake into the cells.

The ultimate goal of the nanoparticles is to deliver drugs directly into the tumour matrix. Lamprecht *et al* reported a particle size-dependent reduction in IC₅₀ and cell viability by etoposide loaded lipid nanoparticles in selected glioma cell lines with the smaller particle sizes; 25 and 50 nm showing the highest IC₅₀ and cell viability reduction (235). The smaller nanoparticles of 30 nm and 50 nm were taken up faster and deeper into the spheroids compared to the 100 nm that were limited to the periphery even after 24 hours of incubation. Similar results were obtained by Tchoryk *et al* when they studied the penetration of 30 nm, 50 nm and 100 nm PGA nanoparticles into HCT116 colorectal spheroids (236). The 30 nm and 50 nm NPs showed similar penetration of 70 and 80% after 2 hours of incubation and an increase to 90% after 24 hours. The 100 nm NPs showed less than 10% penetration after 2 hours and 22% after 24 hours. All the above show the importance of size to tumour penetration by nanoparticles. Further optimization can be done to make the nanoparticles prepared in this project smaller in size in order to improve tumour uptake and delivery into tumour matrix.

The zeta potential reported for PGA nanoparticles has been largely negative. -52 mV for RBITC labelled PGA40%C8 nanoparticles (96), -28 mV and -29 mV for cytosine arabinoside and dexamethasone loaded PGA100%C8 nanoparticles respectively (89) and -31.3 mV and -34.3 mV for etoposide and etoposide phosphate loaded PGA100%C8 nanoparticles respectively (99). The zeta potential of the nanoparticles obtained in this project were -45.4 mV for teniposide-PGA50%TRY, -48.4 mV for etoposide phosphate-PGA85%C18 and -64.7 mV etoposide-PGA85%C18. A look at the structure of PGA (Figure 1-5) shows a free carboxyl group present. A protonated carboxyl carries a negative charge which may explain the

negative zeta potential observed, a property that indicates electrostatic colloidal stability in the nanoparticles (98,199).

4.4.2 Drug loading

The amount of drug loaded into a nanoparticle contributes to the successful usage of the nanoparticles as a delivery system. Nanoparticles can convey drugs to the site of action, but the success of the treatment depends on the ability of the nanoparticles to deliver an effective dose of the active ingredient to the desired site of action.

The low drug loading that has been recorded in past works has been suggested as one of the possible reasons for low clinical translation (237). Hence the need to prepare nanoparticles with improved drug loading. On this project, one of the major goals was to improve the drug loading results, especially of the PGA substituted nanoparticles, by taking a reverse engineering approach to the preparation process. The aim is to see the influence of the drug-polymer interaction on drug incorporation.

Etoposide phosphate; had the lowest drug loading followed by Etoposide drug loading which was slightly higher with 5% drug loading despite being hydrophobic. However, the most hydrophobic teniposide loaded PGA_{50%}TRY showed the highest drug loading (Figure 4-1). These drug loading results for the three drugs, show an apparent correlation between drug loading and drug hydrophobicity which was not seen in the screening studies in the last chapter.

The aim of the screening done was to lead to a new set of polymers that will make better nanoparticles. From the screening result, etoposide phosphate had the most retention in PGA_{85%}C₁₈, greater than etoposide. While for teniposide whose polymer was chosen based on highest retention alone showed the highest drug loading. These results show the

possible role of the extent of drug interaction, evident in the ability of the polymer to retain the drug, on drug incorporation into a polymer. However, the etoposide and etoposide phosphate results show that the drug-polymer interactions may not solely be the controlling factor in determining drug loading. For example, Saadati *et al* reported a drug loading of 7% for nanoprecipitation and 12% for solvent evaporation (193). This may show that other factors such as method of preparation may also play a role in drug incorporation.

To give a context for understanding the significance of the drug loadings obtained in this study, it is necessary to look at both historical results from our group and other groups who have incorporated these drugs into nanoparticles. Previous work in our group has used similar drugs and polymers which the present project builds on. Puri in her work reported a drug loading of 1.5% and 1.9% for etoposide phosphate loaded 100%C8 and 5% Tryptophan substituted PGA nanoparticles respectively (89). This is similar to Ivanov who reported a slightly higher drug loading for teniposide than etoposide loaded PGA substituted polymers (117). Some similarities with the results in this project were observed, the drug loading obtained for nanoparticles loaded with teniposide, the most hydrophobic drug was the highest. The poor solubility of Teniposide in the surrounding aqueous media may, therefore, be the factor here.

Ivanov prepared teniposide loaded particles by nanoprecipitation using different polymers such as poly(caprolactone)-PEG (PCL-PEG)) and poly(decalactone)-PEG (PDL-PEG), poly(lactic acid)-co poly(ethylene glycol methacrylate) (PLA-PEGMA) and poly(caprolactone)-co poly(ethylene glycol methacrylate) (PCL-PEGMA) and PGA substituted polymer PGA40%C18 (117). The drug loading results he obtained was less than 0.6% for all the polymers and PGA40%C18 was less than 0.1%. Compared to all of the above the drug loading obtained for teniposide

formulation prepared on this project is an improvement from historical data.

Table 4-6 shows past drug loading results from PGA nanoparticles. This project used similar polymers and methodology for drug loading and clean-up. The drug loading obtained from the drug-polymer matches were much higher for etoposide, etoposide phosphate and teniposide. This suggests that drug-polymer matching is a useful step to increase drug loading.

Study	PGA substituted Polymers	Drug loading (%)
Present study	Etoposide-PGA85%C18	5
	Etoposide phosphate-PGA85%C18	3.5
	Teniposide-PGA50%TRY	13.4
Ivanov 2014 (117)	Etoposide-PGA100%C8	0.05 – 0.06
	Teniposide-PEGPGA	0.1 – 0.2
Puri 2007 (99)	Etoposide-PGA100%C8	3
	Etoposide phosphate-PGA5%TRY	1.9
	Etoposide phosphate-PGA100%C8	1.8

Table 4-6 Summary of historical drug loading data for Etoposide, Etoposide phosphate and Teniposide loaded PGA substituted nanoparticles via nanoprecipitation. The drug loadings obtained in this study are higher than historical data.

In the literature, reported drug loading for teniposide formulations are as follows. TENI loaded multilayer modified albumin nanoparticles were also reported to have a drug loading of 2.24% (238). A higher loading of 4.29% was achieved with albumin nanoparticles loaded with teniposide (234). The highest drug loadings found in reviewed works were 12.4% and 18.53% which was reported by Mo *et al* when they prepared loaded PLGA nanoparticles loaded with teniposide and Chu *et al* formulation of teniposide loaded into monomethoxy poly(ethylene glycol)-poly(ϵ - caprolactone-co-D,L- lactide) (MPEG-PCLA) polymeric micelle respectively (190,239).

In preparing nanoparticles, a vital step is the removal of unincorporated drug (see section 2.2.2.2). Ineffective removal of unencapsulated drug might lead to exaggerated drug loading values. Some reported drug loading in the literature could be lower if the nanoparticles were properly cleaned. For example, Wang *et al* reported 8.5% loading for etoposide loaded bovine serum albumin nanosuspension, however, there was no clear clean-up description to remove unincorporated etoposide (185). Some methods that have been used to remove unincorporated drugs include centrifugation (240), size exclusion chromatography (117,241) and dialysis (242,243). In this project, a size exclusion chromatography method was used to clean up nanoparticles and a way to confirm removal was via the attached UV recorder which showed different peaks for free drugs and nanoparticles. Thus, while some of the drug encapsulation for etoposide was higher than achieved in the present project, there is concern that drug loading in some of these reports may be exaggerated.

4.4.3 Drug release

The cumulative percentage drug release in 4 hours for etoposide - PGA85%C18, etoposide phosphate - PGA85%C18 and Teniposide-PGA50%TRY was 12%, 10%, and 27% respectively. This amount of burst release recorded on this project is low compared to the various reports of high burst release for etoposide and teniposide loaded nanoparticles. On this project, burst release was termed as the drug released in the first 4 hours because there is a clear distinction in rate between the early and later periods of drug release.

A low burst release for the DLPNP may have been due to the high drug-polymer interaction earlier observed which perhaps held the drug back from diffusing out of the polymer matrix. High burst release is common in the literature, for example, a burst release of 70% in one hour was observed for etoposide loaded PLGA-PEG nanoparticles, while the rest of the loaded drug was released in 12 hours (106). Also, more than 80% etoposide was reported to have been released from poly(butyl cyanoacrylate) nanoparticles within 6-7 hours (244). The method of strategically picking the polymers based on screening results may have made a difference in this project.

Drug polymer compatibility was assessed using films and the release results obtained from the nanoparticles were compared with the release from the films.

System	Drug released in 24 hours
Etoposide-PGA85%C18 film	64%
Etoposide-PGA85%C18 DLPNP	22%
Etoposide phosphate-PGA85%C18 film	23.8%
Etoposide phosphate-PGA85%C18 DLPNP	16%

Teniposide -PGA50%TRY film	29%
Teniposide -PGA50%TRY DLPNP	35%

Table 4-7 the percentage drug released in 24 hours from the drug-polymer films from chapter three and the DLPNP prepared in chapter four.

From the summary (see Table 4-7), the percentage drug release from the etoposide phosphate and teniposide nanoparticles were quite low and closer to the release from the films than etoposide – PGA85%C18. It appears that for etoposide phosphate and teniposide the films were a fairly good predictor of the release from the nanoparticles. The choice of PGA50%TRY was based solely on release from the films and this may have had an impact on the release profile of the PGA50%TRY nanoparticles as the release from the Teniposide DLPNP formulation was the closest to the film. For etoposide PGA85%C18 the higher drug release may have been due to the fact that the chosen polymer was based on the other factors (contact angle and FTIR) in addition to drug retention in the film.

The diffusion of drug into the dialysate from the drug spiked empty polymer nanoparticles was monitored as a control however, the pattern of drug diffusion observed was interesting. For etoposide where double the amount of the drug diffused from the drug-spiked PGA85%C18 nanoparticles in 4 hours and the rest of the drug diffused more slowly afterwards. This shows an unexpected association of the free etoposide with the polymer which prevented the drug from diffusing out of the dialysis bag. This is believed to be due to the extent of interaction occurring between the released drug and the polymer which has led to the adsorption of the drug to the polymer. This may be a reflection of the high compatibility between etoposide and the chosen polymer PGA85%C18. Such a high affinity of the drug to the polymer could lead to a different way to load drug nanoparticles, by incubation of pre-formed nanoparticles with the drug. This was however not explored in this project.

The results from the control in the case of Etoposide phosphate and teniposide showed that they did not associate as much with the polymer as etoposide. From the screening release result, etoposide phosphate was retained more by PGA85%Cl8 which should mean the association with the polymer should be more with etoposide phosphate. However, this was not the case. A possible explanation may lie in the difference in the solubility of the drugs in the dissolution media which may interfere with the adsorption process. Also, a possible explanation for the low adsorption by Etoposide phosphate may be due to its higher solubility in the dissolution media which may account for a higher opposing force pushing it out of the dialysis membrane than the adsorption to the polymer.

The release of etoposide, etoposide phosphate and teniposide from the nanoparticles in this study appeared to be incomplete. The favourably high drug retention after 48 hours is believed to be a consequence of the high compatibility already demonstrated between the drugs and the chosen polymers. The high amount of drug retention required on this project makes incomplete release desirable. The explanation Gaucher *et al* gave for the incomplete release of paclitaxel from Poly(D,L-lactide) (PDLLA) amphiphilic block copolymers was high compatibility between drug and polymers (240). In Meng *et al* a localisation of RBITC labelled PGA nanoparticles in the endosomal/lysosomal compartments of DAOY cells was observed when the uptake and metabolism of PGA nanoparticles in DAOY cells was monitored (96). This study shows a possible fate for the nanoparticles on this project. Upon uptake into the tumour, drug release of the formulations is expected after degradation of the polymers in the lysosomes. So, high drug retention due to high drug=polymer compatibility would ensure the nanoparticles for uptake retain as much of the loaded drug as possible at the time of uptake.

Simulating the expected conditions of the *in vivo* target site is important when setting up *in vitro* release experiments. In setting up the *in vivo*

release experiment in future the actual condition of the tumour microenvironment should be taken into consideration. The degradation of the polymer is also a key factor which is expected to occur by the rapid action of lysosomal enzymes (96). Release experiments in the presence of enzymes should be considered in future experiments to address this parameter.

4.4.3.1 Drug release modelling

A zero order release rate would mean that a constant amount of drug was released from a matrix that does not disaggregate and this release is independent of drug concentration (130). A first order release rate indicates that drug release is concentration dependent and it can be applicable to sustained release from a matrix system (111). Higuchi release rate means that the drug release is from a matrix and the diffusion of the drug is based on Fick's law. It also assumes no alteration of the matrix structure as it comes with water. The other assumptions of the Higuchi model are stated in section 1.4.5.2.2. Lastly, Korsmeyer Peppas release rate is applied to the release of drug from a polymeric matrix system, whose mechanism of drug release is unknown (130).

The *invitro* data of the release experiment was fitted to these models above and the graphical representation (see Figure 4-5, Figure 4-6 and Figure 4-7) and correlation coefficient (r^2) values were presented in Table 4-3. The values of the zero order correlation coefficient were low in all the three formulations. The first order was the highest for etoposide-PGA85%C18 and etoposide phosphate this may indicate that the release of the drug is dependent on its concentration. The Higuchi model correlation coefficient value was high for etoposide-PGA85%C18, however, for teniposide-PGA50%TRY, no other model correlation value was higher than Higuchi. The values of the correlation coefficients are not high

enough to state that any of these models will fully explain the drug release. However, the Higuchi values show that diffusion may be a key factor.

The release mechanism is driven by Fickian diffusion when n is equal to equal to or lower than 0.43 for spherical particles (245). The exponent of release (n) of Korsmeyer-Peppas model obtained for these formulations are stated in Table 4-4. These values indicates that Fickian diffusion may have been involved in the drug release of etoposide and etoposide phosphate DLPNP. However, the n value of teniposide DLPNP is above 0.5 which indicates non-Fickian diffusion.

4.4.4 Cell culture

The results from the MTT assay were presented as dose response curves. The aim is to compare the cytotoxicity of free drugs to the DLPNPs and to see how the drug loading and retention affects the cytotoxicity of the various nanoparticles.

Looking at the curves the free drugs and the nanoparticles showed a reduction in the viability of UW228-3 cells after 24 hours exposure. Etoposide PGA85%C18 appeared to be more cytotoxic than free etoposide, evident in the slopes of the dose response curves and consequently the IC₅₀ values. Etoposide phosphate PGA85%C18 and Teniposide PGA50%TRY were observed to be less cytotoxic than their free drugs. Evident also in the dose response curve and the IC₅₀ obtained. A higher IC₅₀ of 41.6 μ M was obtained for teniposide nanoparticles compared to free teniposide which was 16 μ M while that of etoposide phosphate-PGA85%C18 was almost 10 times higher than that of the free drug. Comparing the cytotoxicity of free drug with that

The dose response curves all showed a relatively high cell viability at high drug concentrations. It looks like some population of the cells used in the

study appear to become resistant to the treatments used. This may be due to a slower growth of the cells or lower ability to take up the drugs. However, as all treatments and controls were carried out under identical conditions, the comparisons between the free drugs and the formulations should still be valid.

Ideally, for application to *invivo* studies and clinical work, other factors may affect the absolute cytotoxicity results given. Furthermore, there is some evidence of the impact of exposure time on the IC₅₀ obtained from cytotoxicity studies. A previous study by Athawale *et al* where they prepared etoposide loaded solid lipid nanoparticles (etoposide-SLN) and compared their cytotoxicity on B16F10 melanoma cells with free etoposide (76). Their results showed a time and concentration-dependent cytotoxicity. They obtained an IC₅₀ of 3.4 μ M for free etoposide and 17 μ M for etoposide-SLN after 24 hours exposure time and the IC₅₀ of etoposide-SLN reduced to 3.4 μ M after 48 hours exposure. This gives an indication that the IC₅₀ obtained could be time-dependent and higher exposure time could lead to a lower IC₅₀.

Some of the processes that may affect cytotoxicity include drug release rate, completeness of the uptake of the nanoparticles, rate of degradation of polymer, drug release in lysosomes, solubility of the drug at pH 4 -5.5 and the transport of drug from the lysosomes to the cytoplasm and nucleus. A full understanding of the pathway of the drug from endocytosis up to its accumulation at the target site and the effect of these processes on cytotoxicity. After 24 hours the higher cytotoxicity of the etoposide-PGA85%*C18* compared to the other nanoparticles may have been due to the ability of the etoposide to escape from the lysosomal compartment compared to teniposide despite the higher drug loading, this difference may be due also to the differences in the solubility of the drugs, or possible sequestration or inactivation of the drug before getting to target sites.

The interpretation of the cytotoxicity results for drug delivery can be difficult. Rapid release of drugs from nanoparticles prior to endocytosis might make nanoparticles appear to have cytotoxicity similar to the free drug. However, nanoparticles with more sustained release or higher drug retention will be expected to show a different cytotoxicity pattern compared to the drug. The IC₅₀ of nanoparticles is expected to be affected by drug loading due to the saturable nature of endocytosis, good intrinsic cytotoxicity and good drug retention within nanoparticles before uptake, not cells.

Therefore, the observed differences in the cytotoxicity of the nanoparticles may be better explained when a full assessment of the different factors that may affect cytotoxicity. Further studies have to be carried out to study the mechanisms of both exocytosis, endocytosis and intracellular transport. The impact of the high drug retention, the rate of accumulation at target site and the ease of lysosomal escape on cytotoxicity. The effect of the higher interaction between drug and polymer as it affects the release of drug and the specific advantages to the proposed delivery system. Finally, the rate of drug clearance from the site of action and to compare it with the rate of drug entry and the effect on duration of action. As this project was aimed at solving some of the physicochemical issues affecting low drug loading and drug retention, it was not possible to monitor all the above parameters in this project.

4.5 Conclusion

In this chapter, etoposide, etoposide phosphate and teniposide loaded nanoparticles were prepared with PGA85%C18 for etoposide and etoposide phosphate and PGA50%TRY for teniposide by nanoprecipitation. The particle sizes (diameter) ranged between 111 nm and 214 nm and Zeta potentials of -45 to -65 mV. The drug loading obtained was 13% for teniposide, 5% for etoposide and less than 5% for

etoposide phosphate. The release results obtained were sustained with only a small amount of initial burst release. The cytotoxicity experiment results showed cytotoxicity in UW228-3 MB cells exposed to the drug loaded nanoparticles with etoposide-PGA85% C_{18} being the most cytotoxic. Nanoparticles with improved properties such as higher drug loading than historical data, low burst release and high drug retention were obtained which showed the benefit of initially matching drugs to polymers.

The focus of the next chapter is further development of nanoparticles. This is an attempt to make nanoparticles using a different approach in a bid to further improve the nanoparticles prepared, most especially in terms of drug loading.

Chapter 5

5 Further development of Etoposide and Teniposide loaded nanoparticles: preparation of polymer coated drug nanoparticles

5.1 Introduction

The goal of this project is to make nanoparticles with improved physicochemical properties such as higher drug loading and slower release. In chapter 4 nanoparticles from the drug-polymer matches from chapter 3 (Table 3-6) were prepared using the nanoprecipitation method to produce a matrix type nanoparticle. From the results in the previous chapter, the drug loading obtained for teniposide-PGA50%TRY nanoparticles were the highest followed by etoposide. These drug loading results are higher than previously obtained using PGA nanoparticles, see Table 4-6. The release was also more sustained and some drug was retained in excess of 24 hours in some instances.

5.1.1 Limitations of drug loading for Matrix-type nanoparticles

To further compare previous results, Table 5-1 shows a summary of the properties of etoposide, etoposide phosphate and teniposide loaded NPDS in the literature. The aim of preparing this table is to show the drug loading, drug release, clean-up methods and cell culture results of the DS to give an overview of what is possible for drug loadings with matrix type nanoparticles. It was observed that many authors reported encapsulation efficiency in place of drug loading. Reporting the drug loading would show the actual amount

of drug incorporated. In some cases, the authors that reported drug loading, failed to report the method used to remove untrapped drug, the presence of which potentially leads to an exaggerated drug loading value. The methods that can be used for removing unincorporated drug include gel permeation chromatography, centrifugation, filtration (246). Some of these methods, many used in the reviewed papers, have been shown to be ineffective. For example, the centrifuge and washing method was demonstrated to be unable to remove the adsorbed drug (247). In this project, a gel permeation method was used, described in 2.2.2.2. which is the most effective cleanup method.

Drug release results are also important, especially the extent of burst release, and to maintain the drug within the nanoparticles for a low rate of release. Looking at Table 5-1, we see in general, that drug loading is low, or that the figures given for the drug loading are unreliable. Also, that generally release is rapid with a high burst release. Despite the improved results seen for drug loading of matrix type nanoparticles from the previous chapter, there are clearly quite low limits in terms of possible drug loadings using this type of nanoparticle.

(Ref), Year	Drug	Polymer/Nanocarrier	Particle Size (diameter)nm	Clean up	% Drug Loading	Drug Release
(188), 2013	E	PBCN	162, 225	C/W	6	80% in 6-7 hours
(193), 2013	E	PLGA-PEG	170	C/W	12	N/A
(187), 2014	E	PLGA	105	C	1.45	
(248), 2010	E	PLGA	160	C/W	7.5	55% in 24 hours
(76), 2014	E	LNC	47	C/W	0.3	60% in 12 hours, 24 hours sustained release
(249), 2005	E	LNC	385-421	Column	3	30% in 24 hours, 50% in 58 hours
(235), 2006	E	LNC	25-100	F	0.6 -0.1	60%-75% in 48 hours, 7 days slower release

(250), 2005	E	Tripalmitin	387	C/W	4	NA
(251), 2011	E	LNC	126-91	Ultrafiltration	3.6	NA
(193), 2013	E and Docetaxel	PLGA-PEG	173	C/W	12/7	37% burst release slower release for 72 hours
(252), 2014	E	Albumin nanosuspension	190	N/A	8.5	Initial burst release,
(192), 2010	E	PEG-PSA	1800	C/W	41	pH dependent release
(253), 2012	E	Dextran stearate micelles	133-233	D	30	Burst release
(254), 2011	E	PHBHHx	204	C/W	8.8	20-40% Burst release
(191), 2010	E	PLGA-Mpeg, PLGA-Pluronic	94.02-132.9; PLGA-peg, 148.0-169.9; PLGA-Pluronic	C/W	10	26% in 12 hours, 50% in 48 hours
(189), 2013	E	PLGA-P188, PLGA	PLGA 120-135, PLGA-P188, 115-130	C/W	7.7	60% in 6 hours, 80% in 48 hours

(255), 2012,	E	Mpeg-Ma, Mpeg-Sa, mPEG-Ba	MPEG-Ma-13, Mpeg-Ba-479	C/UF	5	Burst release in 5 hours
(242), 2012	E	LNC	125-170	D	0.38-1.86	Released in 6 hours
(195), 2012	E, Quercetin	PLGA	148.6-153.4	C/W	1.5	12 hours in 40%, 50% in 48 hours
(256), 2013	E	LNC	54	N/A	0.57	40% in 48 hours
(99), 2007	E	PGA100%C8	126	Column	3	40% in one day, 50% in 6 days
(117), 2014	E	PGA100%C8	132	Column	0.05-0.06	Burst release
(190), 2012	T	PLGA	121	C/W	12.37	35% Burst release
(257), 2013	T	TPGS, MCT,	31	C	2.16	pH dependent release
(258), 2016	T	MPEG-PCLA, Micelle	29.6	F	18.53	33% in 8 hours, 78% in 72 hours

(238), 2016	T	Albumin-chitosan-PCL-PEG	260	Ultrafiltration	2.24	Ph dependent release
(234), 2013	T	Albumin	182.3	Column	4.29	
(117), 2014	T	PGA-PEG	162	Column	0.1-0.2	Burst release
(259), 2015	Etoposide phosphate	Indium/theranostic implant	55	C	Not reported	No burst release, sustained release
(99), 2007	Etoposide phosphate	PGA _{100%} C8	142	Column	1.8	30% released in 5 days

Table 5-1 Summary of the properties of nanoparticle delivery systems using etoposide, teniposide and etoposide phosphate. The drug loading, particle size, clean up method and release results are shown. The most commonly used clean up method is the centrifuge and washing. The abbreviations: LNC; lipid nanocapsules, Poly(butyl cyanoacrylate); PBCN, poly(ethylene glycol)-co-poly(sebacic acid) (PEG-PSA), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) PHBHHx. D; dialysis, UF; ultrafiltration, F; filtration, C; centrifuge, C/W; centrifuge and washing.

5.1.2 Polymer coated drug nanoparticles

In this chapter the goal was to attempt to improve on the drug loading results achieved in the previous chapter, using a method that involves preparing nanoparticles of pure drug and placing a thin polymer coat on them. This method is an experimental method that has the potential to massively increase the drug loading of nanoparticles and had just recently started to be investigated within the group. There are many questions to be answered about how the method works and how to fully optimize it. Previous work had used drug nanoparticles created from drug crystals (top-down), but the present project took it a step further to check the possibility of creating bottom-up nanoparticles from drug solutions. Also to check whether the high drug loading results obtained by previous researchers could be achieved with the drugs being used in this project. Full optimization of this method would require a separate study of its own.

5.1.3 Existing studies using polymer coated drug nanoparticles

Coated nanoparticles are useful for the controlled release of drugs, genes and bioactive agents, targeted delivery, prolonged drug action, and protection of the cargo from quick (260). A common example of coating is the preparation of stealth nanoparticles that are able to avoid clearance by the RES, by coating the surface with amphipathic poly(ethylene oxide) (PEO) (138). A vast majority of the studies on NPs coating were based on inorganic NPs such as iron oxide nanoparticles (209). This is one of the first pioneering examples of drug NPs coated with polymers. The aim of a coating is to confer stability, improve dissolution of the drugs or functionality to aid targeting. The coating can also control the size and biodistribution of the nanoparticles (261). A further major advantage is that with a thin layer of polymer coating on a solid drug nanoparticle, the resulting coated drug nanoparticle has a greatly increased drug to polymer

ratio in comparison to the matrix nanoparticles presented in chapter 4 and thus the potential for very high drug loadings.

The choice of materials used as coating is vital to the success of the PCNP. Drug nanoparticles are difficult to prepare, and once created have a strong tendency to aggregate and form larger particles. This can be prevented by coating with a polymer to stabilise the nanoparticles or some time to prevent the further growth of the nanoparticle during preparation. Examples of polymers used include Eudragit (260), HPMC (262), dextran, PEG (193), PLGA, PEO (138) and PVA. A wide variety of different methods have been employed for polymer coatings, but they are typically quite complex and difficult to use or requiring substantial pieces of equipment (260).

Reducing the particle size of poorly soluble drugs by preparing them as nanoparticle is one of the strategies used to improve their solubility and bioavailability (263). It involves preparing nanoparticles of the drugs in the presence of a carrier-free delivery system (264). This is done using two main strategies, the top-down method or the bottom-up method (265). The top-down approach involves breaking down of large particles into nanoparticles by wet media milling or homogenising (266). The bottom-up method, on the other hand, involves the precipitation of dissolved drug (263) and is the strategy used in this project.

Mishra *et al* prepared a nanosuspension of naproxen which when compared to the original crystals of the drug had smaller particles because of the presence of a polymer coating with HPMC (262). Chen *et al* also prepared griseofulvin drug particles coated with eudragit polymer (267). They obtained coated particles without any free drug particle remaining uncoated. They further observed that the drug crystallised first followed by the polymer which then covered the drug thinly, a process called

nucleation which has been studied and forms the basis for producing polymer coated drug nanoparticles.

Looking at the classical theory of nucleation, the multi-component system exists initially as a single phase and any modification such as the addition of an antisolvent changes the free energy of the system (265). This leads to supersaturation and then precipitation of the molecules of the components of the separating phase. A nucleus is formed which continues to grow. There is a critical nucleus radius r^* shown in Figure 5-1.

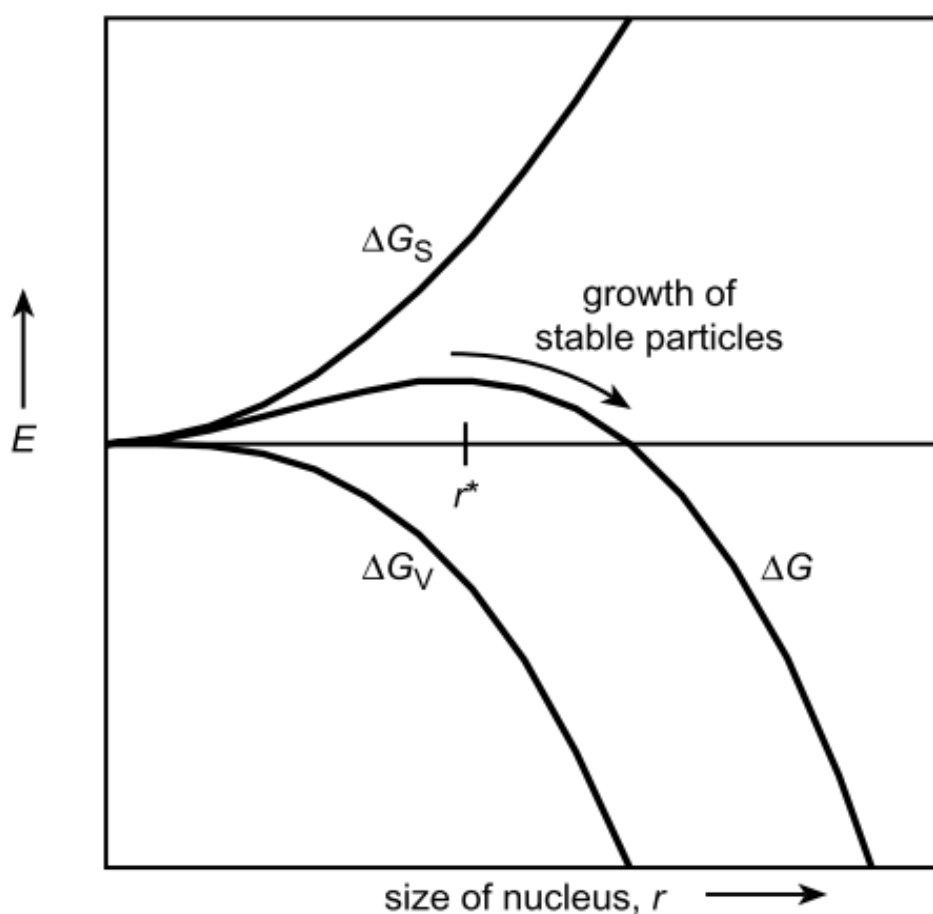


Figure 5-1 The energy diagram of the nucleation process (ΔG : free energy of a particle with radius r , ΔG_s : surface energy, ΔG_v : bulk energy, r^* : radius of the critical nucleus). The particles in the system with $r > r^*$

continue to grow while particles less than r^* re-dissolve. Taken from (265).

The addition of additives for example surfactants and polymers have an impact on the particle formation by the formation of a coating on the forming nanoparticles, thereby controlling their particle size (265). Uncontrolled precipitation may lead to particles sizes similar to the original drug crystals (263) as the nanoparticles will continue to grow forming crystals. Since crystals are more thermodynamically stable, there is a need to stabilize the nanoparticles to avoid large crystal formation by Ostwald ripening which is the deposition of dissolved small particles onto larger particles (268).

The addition of polymers can stop aggregation and stabilize the particles by providing stabilization, which in the case of PEG containing polymers can be a very effective steric stabilization, see Figure 5-2 (269). For crystallization to occur a level of ordered stacking is required which can be inhibited by polymers (270). The effect of the polymers on precipitation, however, depends on the interaction between the forming particles and the polymer (270). This is affected by the functional groups present in both drug and polymer (271). The formation of hydrogen bonds, between the polymer and the drug core, has been stated as the mode of interaction (262). Interactions such as Van der Waals forces also play a role in the adsorption of polymer onto the drug. The stability of the formed particles depends on the affinity of the stabilizer, in this case, the polymer to the drugs and also it must be in sufficient quantity to fully coat the surface of the particle (272). Hence the type and amount of polymer

used is important and the process of selection of such is also important (268).

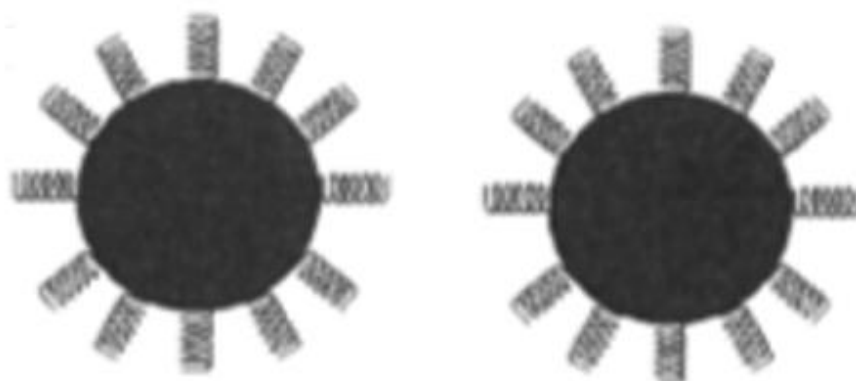


Figure 5-2 Particles stabilised by steric hindrance, adapted from (273).

In the work in the present chapter, hydrophobic etoposide and teniposide are used here because supersaturation has to occur before nucleation starts. A hydrophilic drug like etoposide phosphate will not reach supersaturation upon the addition of the quantity of water used because of its high water solubility.

The pre-matched drug and polymer from chapter 3 were used here largely because of the role of the polymer and the already evidenced interactions between etoposide and teniposide and their chosen polymers. While in most of the previous work reviewed above, the nanoparticles are formed in the presence of polymer, in this project, the polymer coating was added after the formation of the drug nanoparticles. The simple method of interfacial deposition as used here for both the preparation of the drug nanoparticles and the polymer coating process in consecutive steps, exactly the same as for preparation of the matrix nanoparticles in the previous chapter.

5.2 Materials and methods

5.2.1 Optimizing the preparation of polymer free drug nanoparticles

The optimisation of the process of making the drug nanoparticles was done as described in 2.2.4.1.

5.2.2 Preparation of polymer coated drug nanoparticles

Etoposide and teniposide polymer coated drug nanoparticles were prepared as described in 2.2.4.3.

5.2.3 Particle size and Zeta potential of PCDNP

The Z average (diameter) and zeta potential were determined as described in section 2.2.2.3.

5.2.4 Drug loading of PCDNP

The amount of drug loaded into the nanoparticles was determined as described in section 2.2.2.4

5.2.5 Drug release from PCDNP

The cumulative drug released from the nanoparticles was determined as described in section 2.2.2.5.

5.2.6 Cytotoxicity Assay of the free drugs and PCDNP

The cytotoxicity of the nanoparticles and free drugs were assessed using MTT assay as detailed in section 2.2.2.3.

5.2.7 Data analysis

The results were analysed using GraphPad prism 7.0. The concentrations of drug in the samples were calculated and used to plot a graph of cumulative release. The release of the nanoparticles was fitted into mathematical models to further assess the release profile. The experiments were carried out at least three times. The statistical analysis of the cell culture was carried out using GraphPad prism 7.0. The dose response curves were plotted using the same software. The IC₅₀ values were determined from non-linear regression analysis also using graph pad prism 7.0.

5.3 Results

5.3.1 Particle size of the PCDNP

The stirring time (the time during which the solvent evaporates and the nanoparticles 'mature') of the organic solvent containing drug and anti-solvent was varied. The different particle sizes (diameter) obtained for etoposide and teniposide nanoparticles is shown in Table 5-2.

Drug phase	Stirring time	Z average diameter (nm)
Etoposide	0 hour	525.8 $\pm$ 25.4
	4 hours	172.8 $\pm$ 9.4
	overnight	162.8 $\pm$ 2.9
Teniposide	0 hour	188.5 $\pm$ 17.2
	4 hours	155.2 $\pm$ 8.8
	overnight	111.7 $\pm$ 3.0

Table 5-2 Table showing the particle size (z average diameter) of the drug nanoparticles showing the different sizes as the stirring time was varied. The results are a mean of three separate experiments $\pm$ standard deviation (SD).

Organic solvent containing the polymer (0.3 mg and 0.5 mg) was then added to the drug nanoparticles. Etoposide and teniposide drug nanoparticles with polymer coating were obtained and the particle size measured. The results obtained from DLS measurements for the PCDNP are shown in Table 5-3.

Drug	Polymer	NP Particle size diameter $\pm$ SD (nm)		
		Uncoated Drug NP	Polymer Coating	
			0.3 mg	0.5 mg
Etoposide	PGA-85%C18	162.8 $\pm$ 2.9	123.0 $\pm$ 3.4	157.1 $\pm$ 7.5
Teniposide	PGA50%TRY	111.7 $\pm$ 3.0	171.0 $\pm$ 6.2	188.3 $\pm$ 6.4

Table 5-3 The particle sizes of etoposide and teniposide loaded polymer coated drug nanoparticles with 0.3 mg and 0.5 mg polymer coating. The properties were determined using DLS. The results are a mean of three separate experiments $\pm$ standard deviation (SD).

5.3.2 Zeta potential Determination

The zeta potential of the uncoated drug nanoparticles and the coated nanoparticles (0.3 mg) was determined to observe the changes to the zeta potential before and after coating and to confirm that coating has occurred. The different zeta potential values obtained are presented in Figure 5-3.

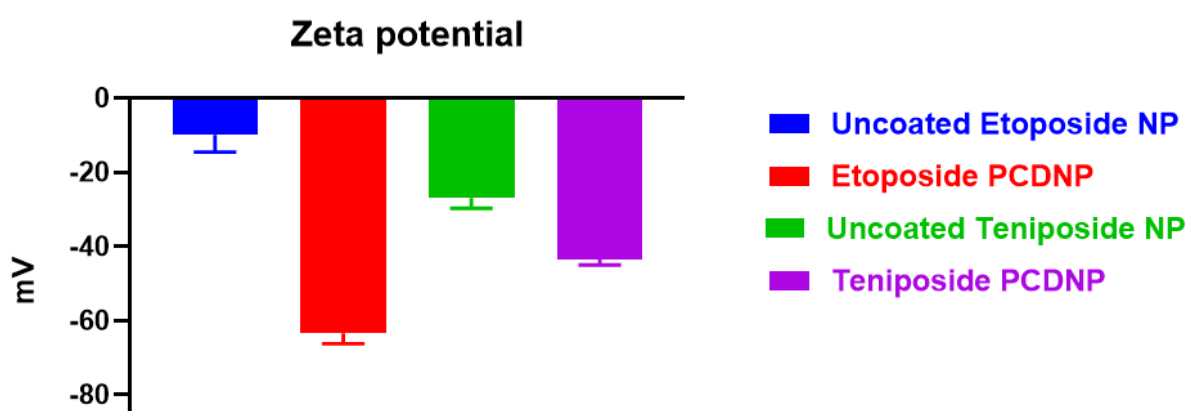


Figure 5-3 Zeta potential of the uncoated drug nanoparticles and the PCDNP. Error bars correspond to the SD of 3 measurements. The coated nanoparticles show higher zeta potential compared to their uncoated drugs.

5.3.3 Drug loading of the PCDNP

Desalting by low pressure gel permeation chromatography was also used here to separate the unincorporated soluble drug from the PCDNP (see section 2.2.2.2). The peaks eluted for the PCDNP were completely separated from the aggregated drug peaks as judged by the baseline separation of peaks on the elution profile.

The determination of the amount of etoposide and teniposide loaded into the PCDNP was done by dissolving them in 50:50 DCM: DMSO and determined using fluorescence spectrophotometry as described in section 4.3.3. The etoposide loaded PCDNP with 0.3 mg and 0.5 mg polymer coating had 76% $\pm$ 10 and 65% $\pm$ 5 drug loading respectively, while teniposide loaded PCDNP with 0.3 mg and 0.5 mg polymer coating had 31% $\pm$ 9 and 25% $\pm$ 5 respectively. The results are the mean of three separate experiments $\pm$ standard deviation.

5.3.4 Drug Release from PCDNP

The drug release experiments for the nanoparticles was carried out using dialysis. The frozen and thawed samples were analysed using the validated HPLC described in section 2.2.1.1.2. Nanoparticle suspension samples were placed in the dialysis tube while maintaining sink conditions. Free drug in a dialysis tube and empty nanoparticles were spiked with the same amount of drugs to compare the release of free drug and the interaction between free drug and polymer.

Figure 5-4 shows the cumulative release of etoposide from ETO-PCDNP and etoposide spiked empty PGA-85% C_{18} nanoparticles and the diffusion of free etoposide. About 10% and 12% Etoposide was released from etoposide-PCDNP after 4 and 48 hours respectively. The release from the spiked nanoparticles was about 27% and 35% after 24 and 48 hours

respectively. Fifty percent of free etoposide diffused out of the dialysis membrane after 4 hours and the free drug release was complete by 48 hours.

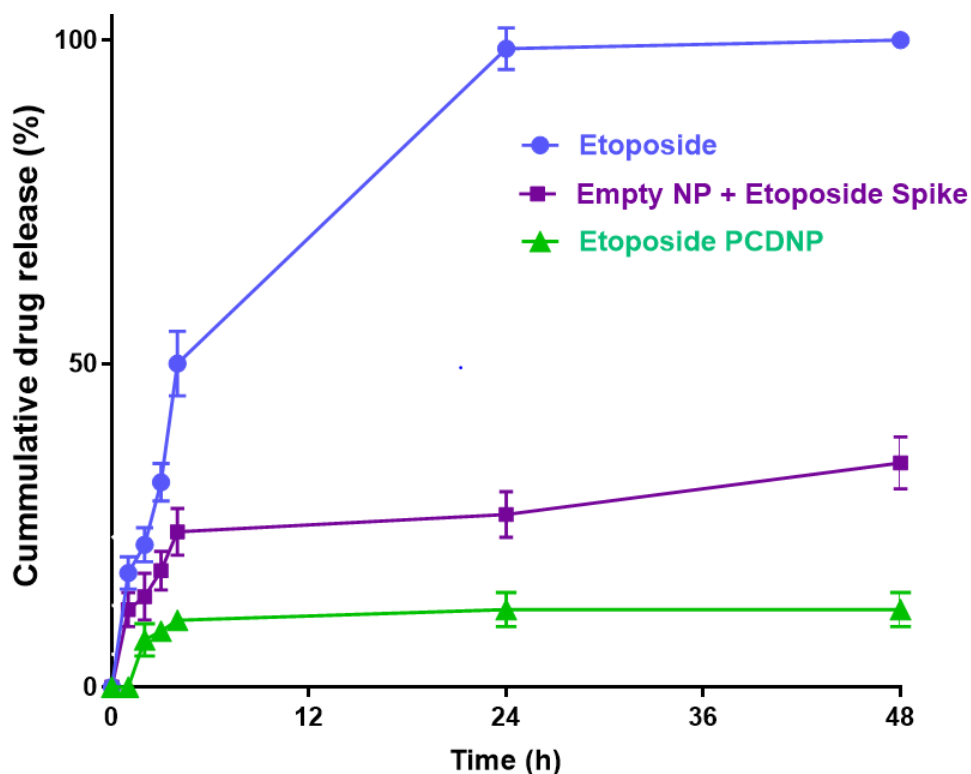


Figure 5-4 Release from etoposide loaded PCDNP with 0.3PGA85%C18 polymer coating determined by dialysis. Also showing the rate of diffusion of free drug into the dissolution medium and release from etoposide spiked empty nanoparticles.

The cumulative release of free teniposide from teniposide-PCDNP and teniposide spiked PGA50%TRY empty nanoparticles and the diffusion of free teniposide after 48 hours is shown in Figure 5-5. About 50% burst release was observed from all three and nearly 100% of free teniposide was released by 24 hours and was complete after 48 hours. Teniposide PCDNP released 63% teniposide after 24 and the teniposide spiked empty nanoparticles released 62% after 24 hours. 72% and 83% of teniposide was released from teniposide-PCDNP and teniposide spiked empty nanoparticles after 48 hours.

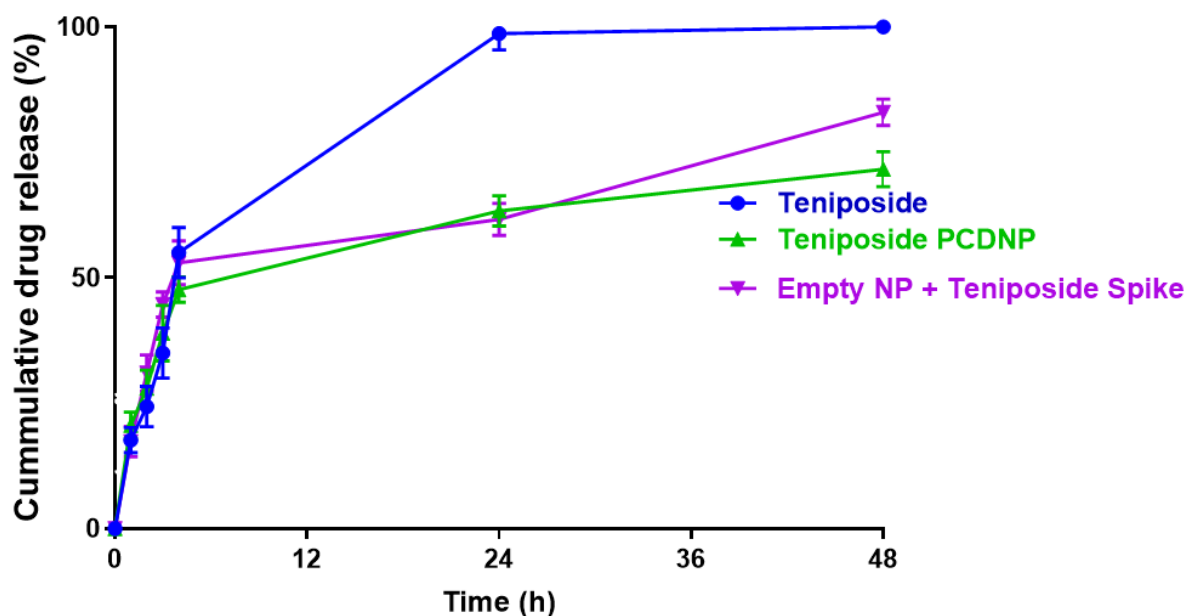


Figure 5-5 Release from teniposide loaded PCDNP with 0.3PGA50%TRY polymer coating determined by dialysis. Also showing the rate of diffusion of free drug into the dissolution medium and release from teniposide spiked empty nanoparticles.

5.3.4.1 Drug release modelling

The expected mechanism of release from the PCDNP is different from the DLPNP. Zero order, first order, Higuchi and Korsmeyer Peppas models were fitted. Figure 5-6, Figure 5-7 and Figure 5-8 are the Higuchi, first order and Korsmeyer Peppas plots of the PCDNPs. The kinetic models did not give a linear relationship and the R^2 values obtained (Table 5-4) were all low, however, the highest R^2 value obtained were for the Korsmeyer Peppas model plot.

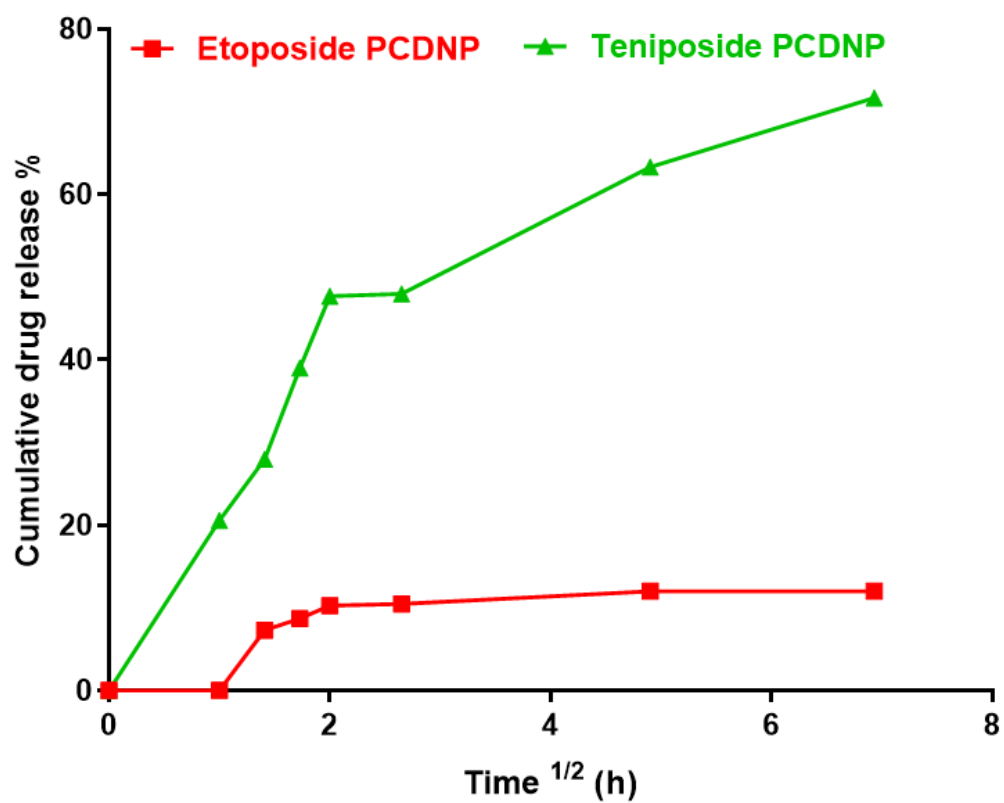


Figure 5-6 Higuchi plots for etoposide PCDNP and teniposide PCDNP.

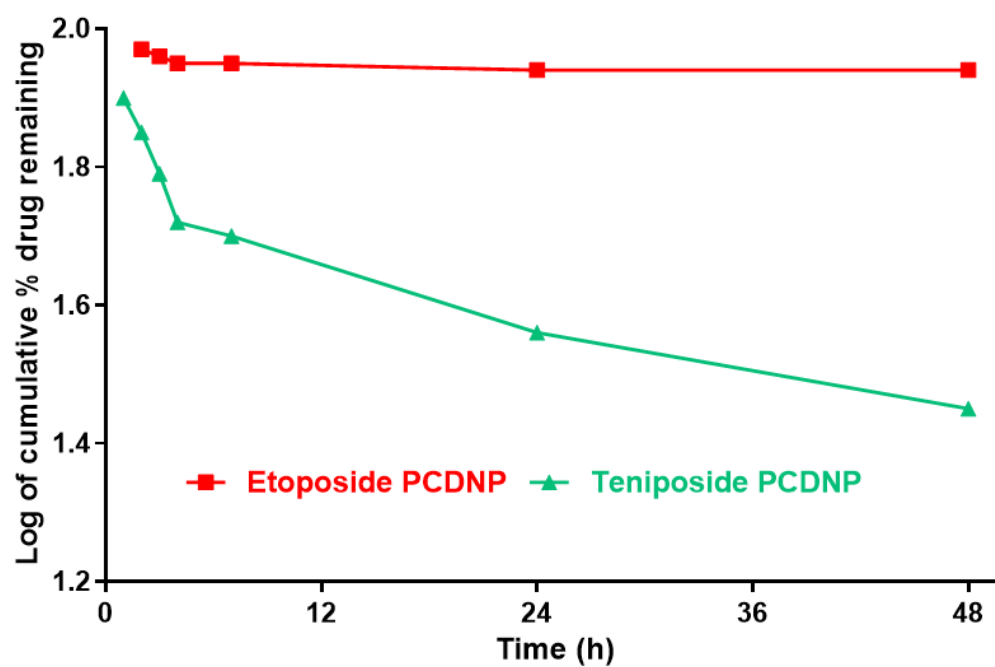


Figure 5-7 First order model plots for etoposide PCDNP and teniposide PCDNP.

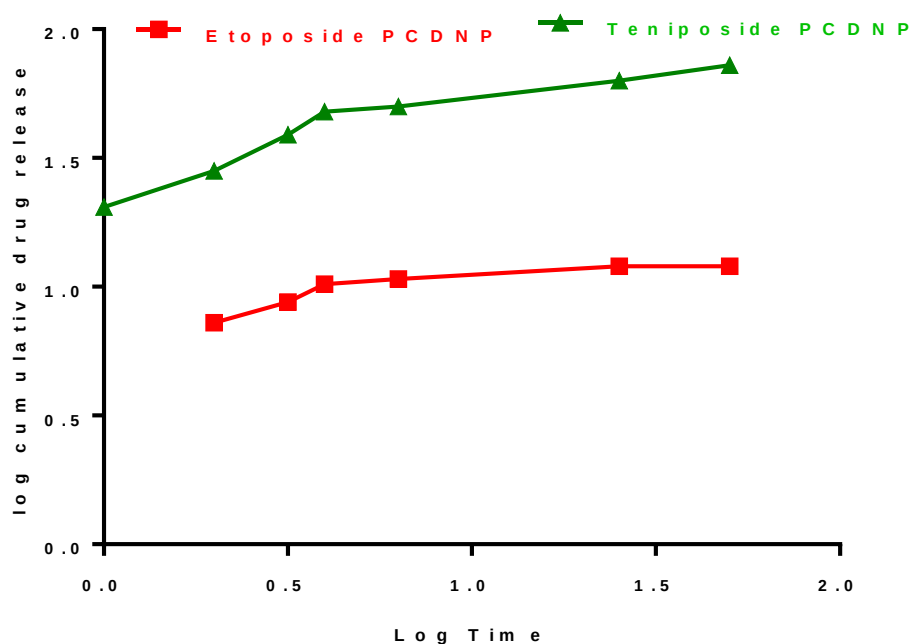


Figure 5-8 Korsmeyer Peppas model plots for etoposide PCDNP and teniposide PCDNP.

Model	Etoposide PCDNP	Teniposide PCDNP
Zero order	0.402	0.6554
First order	0.5571	0.8513
Higuchi	0.5956	0.8474
Korsmeyer Peppas	0.8107	0.8924

Table 5-4 Table of the R square value of the linear regression of the plots of various drug release models of the polymer coated drug nanoparticles. None of the models show a useful correlation for the release of the PCDNP.

5.3.5 Cytotoxicity Assay

The dose-response curves obtained for etoposide and teniposide and their PCDNP formulations are shown in Figure 5-9 and Figure 5-10. The dose response curves show that the etoposide PCDNP is more cytotoxic than free drug but only a little less cytotoxic than etoposide DLPNP. The etoposide PCDNP, however, has cells viable at high drug concentration. In the case of teniposide, teniposide appears more cytotoxic than the teniposide PCDNP. The IC_{50} obtained from the curves are shown in Figure 5-9 and Figure 5-10. The IC_{50} values (Table 5-5) were 20.5 μ M and 6.5 μ M for free etoposide and etoposide-PCDNP respectively and 16 μ M and 35 μ M for free teniposide and teniposide-PCDNP respectively.

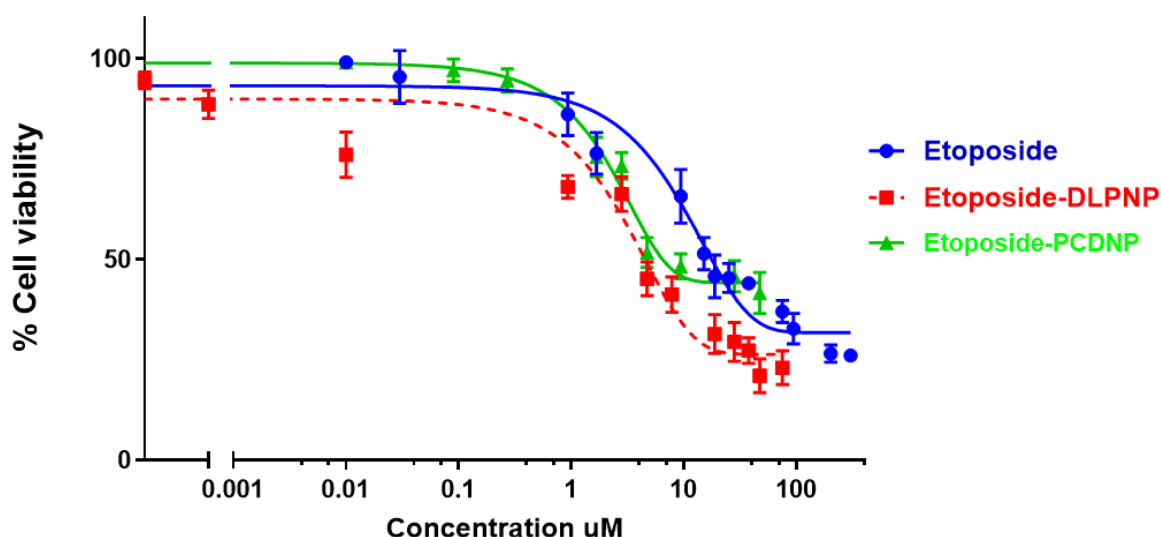


Figure 5-9 Dose response curve obtained from MTT assay conducted to assess the cytotoxicity of free etoposide, etoposide-PGA85%C18 and etoposide-PCDNP. (etoposide-PGA85%C18 (red dotted line) has been added for comparison).

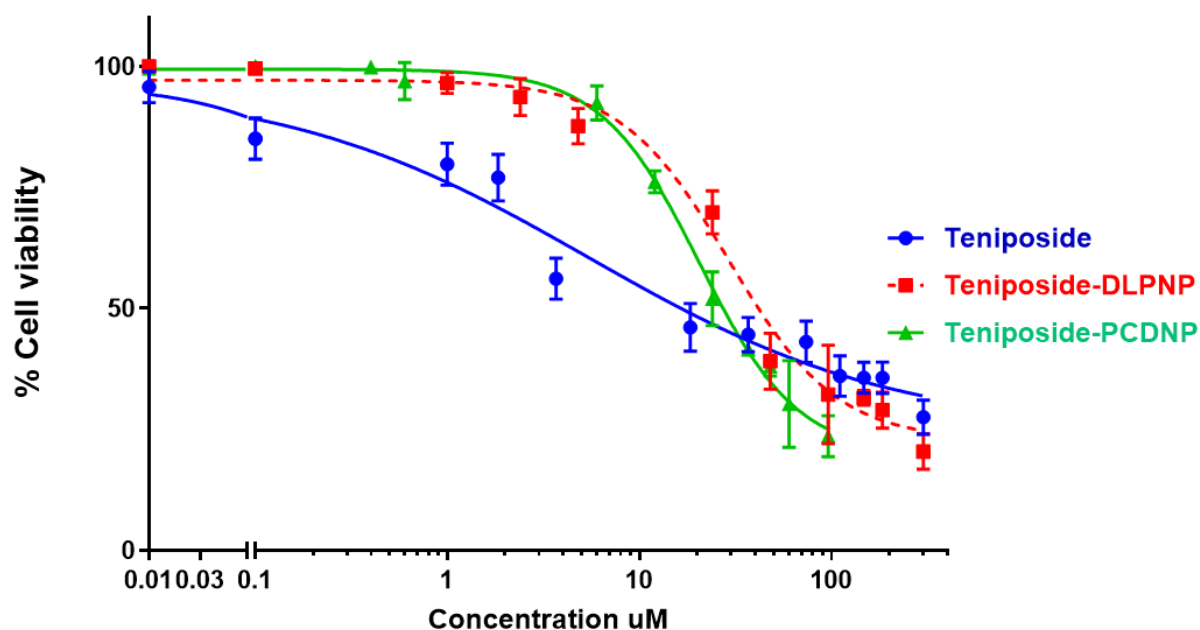


Figure 5-10 Dose response curve obtained from MTT assay conducted to assess the cytotoxicity of free teniposide, teniposide-PGA50%TRY and teniposide-PCDNP. (teniposide-PGA50%TRY (red dotted line) has been added for comparison).

Drug	Etoposide		Teniposide	
IC ₅₀ (μM)	20.5		16.0	
Polymer	PGA85%C18		PGA50%TRY	
Encapsulation	NP	PCDNP	NP	PCDNP
IC ₅₀ (μM)	5.0	6.5	41.6	35

Table 5-5 Table showing the IC₅₀ of the free etoposide and teniposide and the two systems prepared (PCDNP and DLPNP), The IC₅₀ of the Etoposide PCDNP is the lowest overall while the IC₅₀ of the teniposide DLPNP is higher than that of the teniposide PCDNP.

5.4 Discussion

5.4.1 Particle size and zeta potential

The particle size was determined as the maturation time increased. The aim of doing this was to determine the best time to add polymer after particle formation to achieve the best particle size. There are various processes which could be ongoing which may result in either an increase (e.g. aggregation) or decrease (e.g. maturation, condensation, crystallisation) in drug particle size prior to coating. The sizes obtained overnight for drug nanoparticles appeared to be more stable, which is the longest time used before coating. The addition of the polymer leads to disaggregation of the drug particles aggregates leading to the formation of individual coated drug particles. This is similar to the work reported by Styliari from our group using mPEG-PCL coated indomethacin particles and which formed the basis of the present work (274). The final particle size depends on the thickness of the coating formed on the drug nanoparticles.

Upon the addition of the polymer, the particle size of the nanoparticle with 0.5 mg polymer coating was higher than the 0.3 mg polymer coating as expected if all the polymer bound to the drug nanoparticles. That disaggregation occurred with the etoposide-PCDNP was showed by the smaller sizes of the coated drug nanoparticles. However, the teniposide PCDNP were larger than the uncoated drug nanoparticles. A possible explanation for the increase in the size of the teniposide PCDNP compared to the uncoated may be that the drug nanoparticles formed here were colloiddally stable which only increased in size upon the addition of the polymer coating. Also, the calculation used to determine the percentage of polymer added was based on 100 nm nanoparticle. A more tailored

calculation based on the nanoparticles made will be more useful and may yield better results.

The expected particle size reduction would also happen only if the quantity of polymer added is sufficient to coat and stabilize the nanoparticles but not so high as to cause an increase in coating thickness and hence particle size if the amount added is higher than required. If the polymer is added in low quantity, particle aggregation does not stop (269) and also, larger particles may be formed by Ostwald ripening (268).

In the literature, polymer or stabilisers were usually added during drug nanoparticle formation which could lead to less aggregation. This difference in mechanism could lead to differences in the particles formed. Mohyeldin *et al* demonstrated a reduction in particle size with increasing mixing time (275). A similar observation was made on this project as the particle size of the etoposide and teniposide nanoparticles reduced with increasing mixing time from 0 hour to 4 hours and then overnight. The drug nanoparticles that formed overnight were used for further experiments as their particle sizes were the lowest.

A size reduction was similarly observed by Mishra *et al* when they prepared naproxen nanosuspensions stabilised using hydroxyl propyl methylcellulose (HPMC) (262). They equally observed an increase in particle size also as the polymer percentage increased. The polymer acts as a stabiliser to reduce particle size however the explanation for a higher particle size with higher concentration given was that the higher polymer concentration resulted in a high viscous polymer solution which may serve as a hindrance to diffusion between the solvent and antisolvent. Particle size increase due to Ostwald ripening can be reduced or eliminated if formulation parameters, such as choice and amount of stabilizer, are controlled (52).

The choice of stabilisers contributes to the size of these nanoformulations. Surfactants are some of the stabilisers used in the preparation of drug nanoparticles (271). The following are some of the varying sizes obtained with different stabilisers. Hong *et al* showed that polymer stabilised nanoparticles gave nanoparticles with smaller sizes than surfactants (266). They prepared and characterised myricetin nanosuspensions stabilised with polymers like HPMC E₃, TPGS and hydroxypropyl- β -cyclodextrin (HP- β -CD, and surfactants like sodium lauryl sulphate, poloxamer 188 (P188) and soya lecithin. The HPMC stabilised nanosuspension was the smallest of the preparations. These changing sizes due to differences in stabilisers used, show a need for a drug- polymer selection criteria which further supports the hypothesis on this project about the role of drug-polymer affinity and the need for a selection step.

As earlier stated the nucleation process is influenced greatly by supersaturation which depends on the solubility of the drug in the organic solvent. In addition to solvent type, solvent flow rate also influences particle size and PDI. Mohyeldin *et al* showed a particle size reduction from 327 nm to 173 nm and reduction in PDI from 0.502 to 0.21 for Rifampicin nanosuspensions (275). This is similar to the results obtained by Kakran *et al* who observed a particle size reduction from 255 nm to 170 nm as the solvent flow rate increased from 2 ml/min to 8 ml/min (276). In this project, the solvent rate was not varied, however, there were changes in the particle size of the drug nanoparticles as the mixing time increased.

The amount of information in the literature on the specific guidelines on the type and amount of drug and excipients needed in the preparation is limited (269). Further optimization of the preparation process can lead to more control of the distribution and possibly smaller size of the particles. In addition to this, the morphology of the different particles should be studied to see the coating pattern and the difference between the coating ability of the different polymer percentages. A more tailored calculation

based on the nanoparticles made would be more useful and may be a better starting point to finding the optimum amount of polymer to drug required.

The zeta potential values obtained were higher for the PCDNP compare to the uncoated drug nanoparticles. The presence of a thin layer of polymer coating containing a protonated COOH functional group led to the higher negative zeta potential values.

5.4.2 Drug loading

The percentage drug loading obtained for the PCDNP coated with 0.5 mg polymer coating were lower than those coated with 0.3 mg for both etoposide and teniposide. Since the particle size of the 0.5 mg PCDNP was larger than 0.3 mg, the characterization was continued with 0.3 mg. The amount of polymer added to stabilise the nanoparticles also affects drug loading and the ability of nanocrystals to form. Liu *et al* prepared Paclitaxel nanocrystals stabilised using pluronic F127 polymer and reported 20% drug loading when they used a drug: polymer ratio of 1:4, which was higher than previously prepared Paclitaxel formulations (277). They observed changes in the drug loading with different drug polymer ratio. At a drug: polymer ratio of 1:3 no nanocrystal was formed.

The % drug loading of the etoposide-PCDNP was higher than that of teniposide-PCDNP. Compared to DLPNP, PCDNP yielded higher drug loadings. The highest % drug loading overall was obtained for etoposide-PCDNP. High drug loading is not uncommon with this method. Styliari (2017), performed simulations to understand the behaviour of the solvents as they coexist in a system and how their interaction affects the polymers and the drug nanoparticles (274). Experimental and computational work was carried out side by side. She recorded a high drug loading (78.27%) at

low polymer concentrations. The low polymer concentration used in this technique contributes to the high drug loadings obtained.

Preparation of nanoparticles from drug depends on the hydrophobicity of the drugs because precipitation depends on supersaturation. The loading obtained for teniposide-PCDNP was lower than the etoposide-PCDNP despite being the most hydrophobic. This may be that some of the Teniposide had been lost in the process leading to a lower ratio of drug to polymer being present in the PCDNP. Key observations were made by Styliari, which might explain the processes involved in the formation of MPEG-PCL-indomethacin nanoparticles (274). The indomethacin nanoparticles were dispersed in water in which they are insoluble. During the mixing of the indomethacin NP (dispersed in water) and the polymer dissolved in acetone, it was observed that the indomethacin NP remained intact in the first 100 ns. As the drug NP got close to the polymer because of the presence of acetone molecules, more interaction occurred with the polymer chains for more than 150 ns. As the acetone evaporated (overnight) MPEG-PCL-indomethacin nanoparticles were formed with a drug loading of 99.3%.

It is desirable to have a high drug to excipient ratio as this will translate to delivery of a high amount of drug to the target site (277,278) and high drug loading is very common with this technique (271). Overall, the drug loading of 31% $\pm$ 9 and 76% $\pm$ 10 obtained for etoposide-PCDNP and teniposide-PCDNP are considerably higher than the results for many other published results (Table 5-1). The implication of using this technique for the formulations on this project is that the delivery system prepared will have a smaller amount of polymer delivered to the brain compared to DLPNP.

5.4.3 Drug Release

The release experiments were carried out only on PCDNP prepared using 0.3 mg PCDNP since the 0.5 mg PCDNP had been excluded based on their larger size and lower drug loading.

The amount of etoposide released from the dialysis membrane was low even after 48 hours, particularly compared to the free drug, and minimal burst release was observed. The diffusion of etoposide from the drug spiked control was delayed too. In the case of teniposide PCDNP, a higher amount of drug was released after 24 and 48 hours. The burst release was higher and the release from the teniposide spiked control appeared to be similar to the PCDNP after 24 and 48 hours.

The release of drug from the PCDNP depends on the rate of diffusion of drug through the thin polymer coat which can only occur after the solvent has diffused across the coating and started to solubilise the drug. The 100% release of the free drug from the dialysis bag after 24 and 48 hours shows that the thin hydrophobic coating on the drug nanoparticles played some role in delaying the drug release. The observed higher percentage of teniposide release after 24 and 48 hours may have been due to the lower drug content of the teniposide PCDNP compared to the etoposide PCDNP.

The drug release from the teniposide spiked control was similar to that of the PCDNP and shows that the diffusion of drug out of the dialysis bag was hindered. This is an indication of some adsorption of drug to the polymer which is probably due to interaction between the drug and polymer. It is important to note, however, that the amount of polymer available for drug adsorption is smaller compared to the DLPNP. The etoposide PCDNP released less drug in 48 hours than the etoposide DLPNP while the reverse was the case for teniposide PCDNP which released more drug in 48 hours than the teniposide DLPNP (see Table

5-6). It could be speculated that the etoposide PCDNP was probably a more stable and consistent large hydrophobic entity that took longer for water to penetrate. The teniposide PCDNP with the lower drug loading and smaller particle size probably has more than one population and with some poorly coated drugs lying outside the polymer coating which are more accessible and released quickly.

System	Burst release (drug release in 4 hours)	Drug released in 24 hours	Drug released in 48 hours
Etoposide DLPNP	12%	22%	34%
Etoposide PCDNP	10%	12%	12%
Teniposide DLPNP	27%	35%	48%
Teniposide PCDNP	48%	63%	72%

Table 5-6 Summary of the drug release results of the drug loaded polymer nanoparticles; showing the burst release in 4 hours and the cumulative drug release in 24 and 48 hours.

The technique used in this chapter has mainly been used in studies in the literature to improve the dissolution behaviour of hydrophobic drugs by increasing their surface area as a result of reduced particle size (264,279). Normally, when this technique is used the polymers are usually hydrophilic and would aid dissolution. However, the addition of hydrophobic polymers such as the ones used on this project would lead to the formation of a compact coating on the nanoparticles. An additional role of the thin polymer coating here is specifically to protect the drug nanoparticles from dissolution by delaying the access of water to the drug nanoparticles.

The rate-limiting step for drug release from a matrix system is usually the diffusion of drug in the matrix. For microcapsules, drug release is by degradation of the polymer (280). The fitting of the models done and the low correlation values clearly show that the diffusion of drug for these polymer coated preparations does not occur in the usual manner. The experimental methods were fitted to zero order, first order, Higuchi and Korsmeyer Peppas models. These models are mainly used for matrix systems and a high level of correlation was not expected for them. The results obtained suggests that diffusion through a polymer matrix does not play a significant role in the release of drug from the PCDNPs.

A better understanding of the various processes involved in the release from this type of system would be required to fully determine the mechanism of release and the choice of model (113). In the literature, novel models have been used to understand the mechanism of release from polymer coated particles and the importance of parameters such as the thickness of the polymer coating and the composition of the coating to drug release. Enslinn *et al* used a fitting software and obtained a sigmoid shaped release profile (281). They observed the significant contribution of the polymer coat thickness and the polymer blend ratio to drug release.

To fully understand drug release from these polymer coated nanoparticles and to choose the right model for these PCDNP more details about the key parameters are required. The dissolution behaviour of both drug and polymer needs to be understood (113). For a DLPNP, factors such as the rate at which water enters the polymer, the solubility of the drug in water, the diffusion of drug through and out of the polymer matrix. However, for the PCDNP, the factors to consider here are the thickness of the polymer coating, the diffusion of water into the membrane the dissolution of the drug and the diffusion of drug out of the membrane.

In addition to this, the behaviour of the polymer upon contact with the dissolution media, the channels through which the solvent penetrates the polymer to get to the drug and the channel of drug movement out of the polymer. Specifically, for this PGA polymer, which is amphiphilic, the presence of the OH groups on the polymer backbone varying with the extent of substitution, it is expected that the OH part of the polymer will provide a channel for the entry of water through the polymer and also as an outlet for drug release.

The *in vivo* drug release of the intended DS on this project is expected to be different from the *in vitro* mechanism. Drug release is expected to occur intracellularly after polymer degradation where the nanoparticle needs to be intact and hold on to the drug long enough for uptake into the tumour. The extent of interaction between the drugs and polymer and the ability of the polymer to hold on to the drugs may play a role in slowing down drug release, ensuring the drug remains within the NP until it reaches the cellular target site. However, once the NP is taken up by the cell, if the polymer is rapidly degraded, the drug (now present as drug nanoparticles) should then dissolve readily due to its small size.

5.4.4 Cytotoxicity Assay

The MTT assay results were presented here as dose response curves similar to chapter 4. The PCDNP showed a reduction in the viability of UW228-3 cells after 24 hours exposure, same as was observed with the DLPNP and the free drugs, though to varying extent. The teniposide PCDNP showed a higher cytotoxicity than the teniposide DLPNP as evident in the former's lower IC₅₀. However, the free teniposide showed a higher cytotoxicity than the two teniposide formulations. In the case of etoposide, the etoposide PCDNP was slightly less cytotoxic than etoposide DLPNP and the two nano-formulations were more cytotoxic than the free etoposide.

The drug in the teniposide PCDNP may have been more easily accessible than the DLPNP. The difference in cytotoxicity compared with the free drug may be due to the delay in accessibility as the free drug are free to act immediately while the process of nanoparticle uptake and drug release might have delayed cytotoxic action. In the case of etoposide PCDNP, it contains a large amount of drug entrapped in a thin coating which may mean here that within the cells the coating may have quickly degraded to release the drug hence the cytotoxicity after 24 hours.

A reduction of NP cytotoxicity compared to free drug was obtained by Lu *et al*, where they observed a higher tumour inhibition with free Paclitaxel solution compared with two nanocrystal formulations (282). The paclitaxel nanocrystals and transferrin stabilised paclitaxel nanocrystals they prepared showed similar cytotoxicity lower than the free drug solution. Mohyeldin *et al* obtained a lower IC₅₀ of 0.5 mg/ml for free Rifampicin against A549 human alveolar epithelial cells compared to Rifampicin nanosuspension which had an IC₅₀ of 0.8 mg/ml (275). They attributed the lower cytotoxicity of the nanosuspension to a slower uptake by the tumour cells. This shows the possibility of improved cytotoxicity for the teniposide formulations if uptake improves.

Another possible explanation for the cytotoxicity results for the teniposide formulations is the fate within the cells. Endocytotic pathways have been postulated as the uptake route for PGA NP. Meng *et al* demonstrated accumulation of PGA NP stabilised using tween 80 in the lysosomal compartment of DAOY spheroids evident by an increase in the size of the compartment (198). However, it is possible that some nanoparticles may escape the lysosomal compartment. The implication of this is perhaps the PCDNP probably were not all taken up into the compartment where the required lysosomal enzyme degradation of the polymer is expected to occur. Perhaps drug action occurred via slow release in the cells rather than after degradation of the polymer.

The demonstrated faster uptake of PGA nanoparticles by Medulloblastoma cells as seen in 3D cell cultures (198) is vital to the success of the formulations prepared on this project. Only etoposide-PCDNP and etoposide-DLPNP appear to show better cytotoxicity than the free drug. There was no literature comparing DLPNP with PCDNP, the comparisons are between the nano-formulations and free drugs. The results in this project show that the teniposide-PCDNP was more cytotoxic than the DLPNP, while etoposide-PCDNP was only slightly less cytotoxic. Possible reasons for this difference may lie in the structure of the two different formulations and the solubilities of the different drugs.

A problem with nanoparticles is the difficulty to load enough drugs in them and the speed with which the loaded amount is released. High drug loading and quick release mean that in a cytotoxicity experiment the cytotoxicity of free drug was being measured rather than nanoparticles. For the cytotoxicity of nanoparticles with sustained or slower release, the other processes that are involved in the release of drug now control the cytotoxicity. The factors to be considered include the mechanism, efficiency and time of cellular uptake of the NPs, the entry of the NPs into lysosomes, the residence time of the intact NPs in the lysosomes, the mechanism of intracellular drug release, the degradation of the polymer, the process of escape from the lysosomal compartment, the availability of the released drug for action, the solubility of the drug after release. The above questions have to be answered to fully understand the mechanism of action of the nanoparticles and hopefully leading to ways to further improve them.

5.5 Conclusion

In a bid to further improve the drug loading of the formulations on this project, etoposide and teniposide loaded PCDNP were prepared. The technique used mainly for hydrophobic drugs involves the precipitation of drugs by the addition of an antisolvent. The drug loading and retention obtained for etoposide-PCDNP was the highest overall. The cytotoxicity results showed that the etoposide formulations were more cytotoxic overall. The technique used here requires more optimisation for improved results. Variation of parameters and conditions such as choice of solvent, solvent – antisolvent ratio, solvent flow rate and mixing time might all provide more control over the behaviour of the PCDNPs.

Chapter 6

6 Summary, conclusions and future work

The key contribution of this project is the development of a screening method to match polymer to drug based on drug-polymer interaction. Furthermore, a novel method of making polymer coated drug nanoparticles was developed.

A drug is required to accumulate in the tumour long enough to exert its anti-tumour effect while avoiding non-cancerous cells. Many drugs however present with bioavailability, solubility, stability or tumour penetration problems. Encapsulation of drugs like these in NPs provides a useful way to circumvent such problems. Novel drug delivery systems are more efficient at tumour specific delivery which consequently leads to a reduction in side effects (251). Etoposide and Teniposide loaded nanoparticle preparations have been extensively explored and it is clear that they can help overcome many of the problems of administering drug alone. The issue of burst release and low drug loading still remain, limiting the clinical translation of NP research.

The extent of interaction between the drug and polymer affects important parameters like loading and release (218). This project set out to study these interactions and use the knowledge to improve the drug loading and drug release of the nanoparticles.

6.1 Drug-polymer screening

This part of the project was set up based on the hypothesis that strong drug-polymer interactions contributed to drug entrapment in a polymer and the ability of the polymer to control drug release. The interactions between the chosen set of drugs (etoposide, etoposide phosphate and teniposide) and a chosen array of polymers using release experiments. ATR-FTIR and contact angle measurements were used to try to explain the release results.

The release methods showed the affinity of the drug to the polymer. The results from these experiments show that the simple release set up can be used to assess the polymer's ability to retain the drug which is expected to impact its release from nanoparticles. The ATR FTIR measures the changes in the drug-polymer films with respect to changes in the functional group as a result of interactions especially due to bond formation. ATR FTIR results in this project were mainly based on the O-H and carbonyl bond formation. The contact angle, which reflect changes in wettability, also show changes in physical orientation of the drug/polymer as they react in the film. Wettability is greatly influenced by the groups present at the surface. Interactions between drug and polymer bring about changes to the surface groups

Overall the screening results show that the idea that hydrophobicity controls the interaction between drugs and polymer is not correct. It highlights the need for a careful strategy when selecting polymers for drugs when nanoparticles are being made rather than the current approach where no specific strategy is followed when such choices are made.

The different release patterns observed for each drug were not fully explained by the FTIR and contact angle techniques, however, a

combination of the three techniques still provided a useful tool that guides the choice of polymers for each drug.

6.2 Preparation and Characterization of nanoparticles

Chapter 4 shows the outcome of the screening method as observed in the properties of the prepared nanoparticles.

The aim of preparing drugs as nanoparticles is to ensure sustained drug delivery, improved bioavailability, minimising drug toxicity, evasion of the body's defence mechanism, targeted drug delivery and improved therapeutic efficacy. Efforts are being constantly made to improve the properties of nanoparticles formulated for efficient drug delivery. The important physicochemical properties of the nanoparticles, that will be useful for post-surgical local delivery as required in this project are high drug loading and high drug retention.

The focus in chapter 4 was the preparation of nanoparticles from the drug-polymer matches, characterization and cytotoxicity tests. The DLPNP nanoparticles obtained had increased drug loading, low burst release and sustained release after 48 hours compared with historical data especially using similar polymers and methods. This high drug loading and low burst release are particularly of great potential benefit to the DS of interest. Low drug loading, high burst release and premature drug release are known challenges for the use of nanoparticles in drug delivery. The hypothesis that a careful selection is required when selecting drug for polymers is supported by the improved results obtained. This further shows the important role of a screening method to choose the right drug and polymer when making nanoparticles.

6.3 Further development of Etoposide and Teniposide loaded nanoparticles: preparation of polymer coated drug nanoparticles

The key benefit of Chapter 5 is the application of a polymer coating technique to drug nanoparticles in a bid to improve entrapped drug while minimising the amount of carrier used.

The concentration of drug required to eliminate tumour cells are required to be at therapeutic levels. The nano-formulation is required to carry a reasonably high drug amount with as little polymer as possible (278). A modified interfacial deposition method was used to place a thin layer of coating on drug nanoparticles. The PCDNP described in chapter 5, made with the hydrophobic etoposide and teniposide differ from the DLPNP made in chapter 4 in the method of preparation, the drug release behaviour and the cytotoxicity results.

This novel method yielded nanoparticles with much higher drug loading. The high drug loading will ensure a higher amount of drug delivered per nanoparticles administered. This further reduces the possibility of saturating the endocytosis process.

The technique was developed on this project and a lot is yet to be understood regarding the experimental process and the characterization of the resulting nanoparticles.

6.4 limitations

This project has a few limitations. The first limitation is from the choice of polymers, in hindsight if the choice was based purely on release rates it may have yielded even better results. PGA50%SA may have been chosen for detailed work with etoposide and PGA30%CH for etoposide phosphate. These two gave the most interesting release and will be worthy of further investigation. However, the choices in these two cases were based on the combination of the three methods and are a useful representation of the aims of the project.

The cytotoxicity experiments also have their own limitations, firstly, the exposure time of 24 hours may have been inadequate for uptake and release of the nanoparticles. Furthermore, 3D cell cultures to show tumour and normal brain cells will be more appropriate and may show the true behaviour of the formulations. Also, 3D cell culture experiments, which are more representative of *in vivo* tumour environment need to be carried out to assess and ascertain the tumour selectivity, penetration, uptake and cytotoxicity of these formulations. Meng *et al* observed that NPs are taken up better in 3D culture than in monolayer (96). Culturing cells in 3D is more physiologically relevant as it is more representative of the *in vivo* conditions. 3D cell culture results will be more predictive of the *in vivo* tumour behaviour as it accounts for complex cellular functions that are present in real life tissues (283).

6.5 Future work

The mechanism of drug release from the nanoparticles, the cellular uptake and the effect of these factors on cytotoxicity and on performance of the nanoparticles require further study. Improved drug loading results were obtained and the next steps are the necessary things to improve the design process of the nanoparticles. How can these nanoparticles be made better;

Some of the results from the drug release experiments showed incomplete drug release. In order to make the release results more applicable, the drug release of the nanoparticles should be set up to simulate *in vivo* conditions in order to be as close to the *in vivo* release condition. For example, the release of the nanoparticles on this project is expected to occur after degradation in the lysosome which contains enzymes such as hydrolases and proteases that can rapidly degrade biological substances (227,228). Work has started in our group to explore this, Sadie *et al* observed an increase in the release of carboxyfluorescein from carboxyfluorescein coupled with PGA(PGA-CF) in the presence of enzymes (lipase and esterase) (284). Suksiriworapong *et al* added carboxylesterase to their dissolution media during their release experiments to monitor the release of Methotrexate from Methotrexate-PGA NPs (285). They observed an increase in drug release in the presence of the enzyme compared to enzyme free hydrolytic release.

The physiological conditions ideal for the enzymes will also be a useful thing to consider. For example, Wersig *et al* conducted release experiments on indomethacin-PGA conjugates at pH 4.75 and neutral pH. Drug release was higher in the acidic environment compared to the neutral one (286). They further added Lipase from *Pseudomonas* species and drug release accelerated further. Their conclusion was that the

presence of enzymes in an acidic pH contributes to drug release from the PGA nanoparticles.

In this thesis, PCDNP; drug nanoparticles of etoposide and teniposide thinly coated with previously matched polymers PGA85%C18 and PGA50%TRY respectively were prepared. The coated nanoparticles show a lot of promise because of the high drug loadings obtained. As they were prepared using a new technique, further optimisation of the preparation method is required. For example parameters such as quantity of polymer, stirring time and solvent will affect the morphology and size of the PCDNPs.

The results from PCDNP show that higher drug loading can be obtained but more optimization to achieve a smaller size is required. A smaller size can ensure their movement through the ECM. Tchoryk *et al* demonstrated the effect of size, polymer flexibility and surface characteristics on the penetration of nanoparticles into HCT116 colorectal spheroids (236). Size requirements up to 100 nm may be useful in the matrix systems, may still be able to achieve good penetration. The matrix which is softer can squeeze through the gaps in the ECM. The smaller size is required for the polymer coated because it is expected that the ability of the PCDNP to squeeze through the ECM may be less good. This because the bulk of the nanoparticle is solid drug and thus they are harder and so cannot deform so easily.

The cytotoxicity of the nanoparticles and the release of drug from them require some consideration for the presence of a coating that has to first be degraded. The morphology of the coated particles may have contributed to the difference in release and cytotoxicity of the DLPNP and PCDNP. A more comprehensive characterisation of the nanoparticles needs to be carried out to better predict the *in vivo* behaviour of the formulations. Beyond 3D cell culture suggested above, cellular uptake

experiments are vital to the success of these DS. The time and spatial drug release is a key factor here. Polymer coated iron oxide nanoparticles were shown to display unexpected uptake behaviour by Abushrida (209). Recent unpublished work in the group has shown that the particle size of the nanoparticles may have affected these uptake results. The various processes involved in the internalization of nanoparticle up to accumulation at target site have also been stated. Investigations to show the specific uptake mechanisms, endocytotic pathway, accumulation into and exit from the lysosomes, possible exocytosis and clearance for the cells all need to be studied.

Further investigation into the specific endocytic routes of the two systems. How the different pathways contribute to overall cytotoxicity Some of the techniques used include the use of inhibitors of specific endocytic pathways which ensures the impact of shutting down one route is determined and the overall contribution of the route to the fate of the delivery system. Also, studies involving the use of specific markers to track the intracellular pathway of the nanoparticles, such as localization into lysosomes and endosomes. For example, fluorescent Labelling is a simple method used to identify endosomal escape. The prepared nanoparticles are conjugated with small fluorescent dyes and the intracellular distribution of the nanoparticles is monitored. The movement of the labelled nanoparticles can then be traced or quantified using confocal fluorescence microscopy or flow cytometry (96).

Conjugation of other materials such as folate can increase their cellular uptake and overall cytotoxicity. An example of this was demonstrated when the cellular uptake of paclitaxel (delivered by monostearin SLN, folated monostearin SLN and PEGylated monostearin SLN) by A549 cells was monitored (287). The folated monostearin SLN showed the highest paclitaxel uptake after 24 hours. This enhancement of intracellular uptake

and hence cytotoxicity by folate conjugation shows the potential of targeting as a way of improving drug uptake.

When all the above has been done the presentation of the delivery system is also important. A gel has been suggested as a carrier for the nanoparticles (117), the choice of this has to take into consideration the physiology of the brain tumour, CSF and the pharmacokinetics of the nanoparticles and the entrapped drug.

6.6 Overall conclusion

The aim of this project is to make drug delivery systems for local delivery with Medulloblastoma as the brain tumour of interest because it is the commonest childhood brain tumour. Also, because it offers two opportunities to apply targeted chemotherapy, locally at the time of operation and in the treatment of metastasis. An exploration of the assembly of a drug delivery system that can be used to achieve this was done.

The requirements for nanoparticles intended for use in the proposed delivery system are biodegradability and nontoxicity of the polymeric carriers, particle size in the nanometer range, high drug retention, sufficient drug loading and slow releasing capabilities. The project particularly dealt with the physicochemical requirements of the delivery system intended for this project; increase drug loading and achieving high drug retention.

The aim of improved drug loading and reduced burst release was achieved in the two systems made, DLPNP and PCDNP. In addition, a novel method of making nanoparticles with 'high' drug loading was developed. Key information obtained from this project was the importance of a guided screening method to help choose drugs and polymer and how this information can lead to better nanoparticles.

The nanoparticles prepared fulfil some of the set requirements and can be modified to further improve their properties. The key next steps would be to also see how the nanoparticle formulations can be adapted to other drug delivery strategies like CED requiring nanoparticles. Other possible routes of delivery that can be considered include intrathecal, intranasal and intravenously. A nanoparticle carrying enough drug and with good

retention provides more options and can be adapted to some of these routes.

Table 6-1 shows a summary of the properties of all the formulations made in this project. The formulations produced here show some improvement over historical data, especially from similar polymer and drugs. Some of the steps required to take this work further have been shown and the goal of achieving improved drug delivery to residual tumours in Medulloblastoma patients is a step closer.

Drug	Etoposide		Etoposide phosphate	Teniposide	
IC ₅₀ (μM)	20.5		18.0	16.0	
Polymer	PGA85%C18%		PGA85%C18%	PGA50%TRY	
Encapsulation	NP	PCDNP	NP	NP	PCDNP
Diameter (nm ± SD)	191 ± 2.9	123 ± 3.4	214 ± 19.5	112 ± 3.0	171 ± 6.2
Drug Release 48 hour	34.3%	12%	20.0%	48.6%	71.7
Drug loading %	5.0	76	3	23	31
IC₅₀ (μM)	5.0	6.5	174.0	41.6	35

Table 6-1 Summary of the properties of all the formulations prepared in this project. The etoposide-PCDNP appears to be the best formulation having the one of the lowest IC₅₀, highest drug loading and slowest release.

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