School of Pharmacy

Development and Evaluation of Polymeric Nanoparticle Formulations for Triamcinolone Acetonide Delivery

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ABSTRACT

The aims of this study were to develop polymeric NP formulations for triamcinolone acetonide (TA) delivery, from biodegradable and biocompatible hydrophobic polymers, which provide sustained release, prolonged stability and low toxicity, and to assess the toxicity of TA NPs (TA-NPs) compared to TA alone upon BALB/c 3T3 and ARPE 19 cell culture models.

The study involved investigation of three different types of polymers: poly(D,L,lactide) (PDLLA), poly(D,L,lactide-co-glycolide)(PLGA) and methoxy-polyethyleneglycol poly(D,L,lactide-co-glycolide)(mPEG PLGA). Two different methods were studied in the TA-NPs preparation: spontaneous emulsification solvent diffusion and emulsification solvent evaporation methods.

The results show that emulsification-solvent evaporation method was superior to spontaneous emulsification solvent diffusion in terms of yield, loading and entrapment efficiency. TA-NPs synthesised of mPEG PLGA exhibited the smallest particle size, highest efficiency and fastest release of TA, whereas PDLLA produced large TA-NPs with the slowest TA release. The toxicity study revealed that BALB/c 3T3 was more sensitive than ARPE 19 and was concentration dependent in response to 24 hour exposure of either TA or TA-NPs, while ARPE 19 appeared to be less sensitive to the exposure. All NPs were less toxic than TA in all concentrations, in both cell models.

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ABBREVIATIONS

ACN	Acetonitrile
AMD	Age-related macular degeneration
CpG ODN	CpG oligodeoxynucleotide
CTFR	Cystic fibrosis transmembrane regulator
D10	DMEM plus 10% fetal calf serum
DCD	Dialysis centrifugal devices
DCM	Dichloromethane
DF12	DMEM:Ham F12 1:1 plus 10% fetal calf serum
DMEM	Dulbecco's modified Eagle's media
DMSO	Dimethyl sulfoxide
DT	Diphteria toxoid
FESEM	Field emission scanning electron microscope
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
mPEG PLGA	methoxy polyethylene glycol poly(D,L,lactide)
MTC	Minimal toxic concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCO	molecular weight cut-off
NP	Nanoparticle
NPs	Nanoparticles
O/W	Oil-in-water
PBS	Phosphate buffer saline
PCL	Poly-(ε-caprolactone)
PDLLA	Poly(D,L,lactide)
PEG	Polyethylene glycol
PG	Poly glycolic acid
PLA	Poly lactic acid
PLGA	Poly(D,L,lactide-co-glycolide)
PLLA	Poly(L lactide)

PVA	Poly(vinyl alcohol)
RPE	Retinal pigment epithelial
rpm	revolution per minute
RSD	relative standard deviation
TA	Triamcinolone acetonide
TA-mPEG PLGA NPs	TA-NPs using mPEG PLGA
TA-NPs	Triamcinolone acetonide-loaded nanoparticles
TA-PDLLA NPs	TA-NPs using PDLLA
TA-PLGA NPs	TA-NPs using PLGA
Tg	glass transition temperature
UV	Ultraviolet
UV/Vis	Ultraviolet-visible
VEGF	Vascular endothelial grow factor
W/O/W	Water-in-oil-in-water

INTRODUCTION

1.1 Sustained/controlled release systems

The main challenge of developing a successful drug delivery system is to deliver the drug promptly to the target site at a rate and a concentration which can maximise the efficacy and minimise the side effects, in a appropriate period of time [1]. Several parameters, such as bioavailability, biocompatibility, site targeting and drug release profile, play a dominant role in administering the drug [2]. Although conventional formulations have been effective for most of the drugs, there have been obstacles for some drugs to achieve the target site, due to poor physicochemical properties and an incapability to pass through the barriers of the body. Such drugs, especially those which have a narrow therapeutic range and exhibit poor solubility but require long term and localised therapy, would need a continuous administration to maintain the plasma level in the therapeutic range over a period of time [3]. In addition, patient non-compliance with the required administration of the drugs appears to be a significant problem [1]. Therefore, several modifications to drug delivery, such as designing sustained or controlled release products, were made, in order to control frequency of dosing and specificity of the drug. This can lead to improved patient compliance [1, 4, 5].

Sustained release (a term that can be interchangeably used with prolonged release [1]) products have been developed commercially since the late 1940s [6] with the aims of minimising the frequency of administration as well as side effects, locally or systematically. Although sustained release products can enhance patient compliance, these products were only set up with the goal of prolonging the therapeutic duration without any consideration of consistency in terms of drug release. In the late 1960s, the term "controlled release" was used to describe a system which could release the drug in a reliable and constant release fashion [6]. By establishing such strategies as controlling the drug released, modifying the methods to reach the target, and maximising the extent of drug concentration on the target site, the drug efficacy could be improved in a controlled release system.

1.2 Nanoparticles (NPs)

Over the past two decades there has been a variety of controlled drug delivery strategies used to enhance the efficacy of the drug, such as developing soluble macromolecular carriers, micellar carriers, and colloidal micro- and nanoparticulate carriers. Those approaches, especially micro- and nanoparticulate carriers, have become extremely valuable in altering the pharmacokinetic and pharmacodynamic properties of the drug.

Among the various colloidal particulate systems, NPs have attracted much interest in the development of controlled delivery systems. Kreuter [7] defined NPs as "solid colloidal particles ranging in size from 10 nm to 1000 nm (1μm)". The drug can be entrapped in, encapsulated in, dissolved in, adsorbed or chemically attached to, the matrix of the NPs, which can be polymers or lipids.

Since this study will mainly focus on polymeric NPs, only the criteria, characteristic and therapeutic application of polymeric NPs (thereafter referred to as 'NPs') will be reviewed in the following sections.

By incorporating the drug into the polymer matrix and reducing the particle size down to nano-scale, the nature of the drug and its interaction with biological membrane can be modified, leading to the improved delivery of the drug to the target site, which is facilitated by a controlled release. NPs can protect the drug from the unfriendly environment thereby avoiding premature degradation of the drug [8]. NPs are reported to be more stable than liposomes [9]. This delivery system was also reported to be able to minimise the local side effects as well as systemic ones [10]. Moreover, NPs can be an exclusive controlled delivery since it can be bound with the specific ligand for targeting drug actions [11-13].

NPs can be further distinguished as nanospheres and nanocapsules. The illustration can be seen in Figure 1.1. In nanospheres, drugs are dispersed in the matrix structure as a uniform dispersion, whereas nanocapsules are vesicular systems in which the drug can be dissolved or entrapped in the inner core or adsorbed onto the surface of inner vesicles [9].

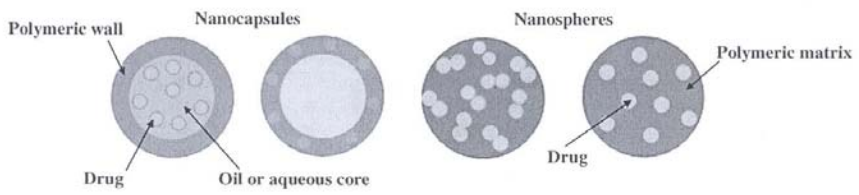


Figure 1.1: Nanospheres and nanocapsules [14].

Compared to microparticles, NPs show significant advantages in site-specific drug delivery. It has been suggested that, for intravenous administration, the particle should be smaller than 8 μm to avoid capillary clogging [3]. In addition, since the diameter of the capillaries is about 5-6 μm, the carriers should be a much smaller size, so they can be delivered without showing any harmful effects for the blood circulation system, such as embolism in the capillaries [12]. NPs also show higher intracellular uptake [9]. NPs synthesised from surface modified polymer, are reported to be able to enhance prolonged blood circulation and pass through the blood-brain barrier [12, 13], therefore, they are potentially useful as an optimised delivery system to intravascular administration.

1.2.1 Criteria of ideal NPs

As a drug carrier system, a nanoparticle (NP) formulation should meet the main criterion, in which the matrix should be biocompatible and biodegradable [3, 15]. The matrix must be non-toxic and must not exhibit any antigenic behaviour to the body [3, 16]. Oppenheim [17] and Domb [18] emphasised that for an ideal drug carrier system, the matrix of the product should be stable during the sterilisation and manufacturing, to enzyme activity, the pH of the environment and must not be degraded in a short period of time and it should provide sustained release. Moreover, the NPs should be able to load the drug, which is either hydrophilic or hydrophobic in nature, in a sufficient amount and maintain the integrity of the drug-matrix before release of the drug at the target site. The matrix, should therefore, protect the drug until it reaches the target site. Finally, the system should provide a reliable and reproducible release kinetics for the drug in a controlled fashion [17]. In terms of drug-carrier production, the cost-effective and reproducibility of the manufacturing of the drug-carrier complex should also be taken into consideration [18].

1.2.2 Limitation of NPs

Much research has been conducted on nanoparticulate system, however it is still in an ongoing process to establish the formulation which is pharmaceutically and clinically accepted. Controlling the particle size has always been a challenge in developing NP formulations. Although nano-sized particles can be easily made by various methods of NP preparation, the physical stability of NPs, mainly the particle size, sometimes cannot be maintained due to the potential risk of particle aggregation. NPs can load either hydrophilic or hydrophobic drugs, however, the level of loading appears to be low, compared to microparticles. In terms of release characteristic, NPs have potential to undergo an initial burst release due to drug adsorption on the particle surface during manufacture. This initial burst release may lead to toxicity if the concentration of drug released is over the minimum level of toxic concentration (MTC) in the blood. In terms of toxicity issue, the various interactions of NPs with biological tissues have to be taken into consideration. NPs may trigger mediator to activate inflammatory or immunological responses. If it is administered intravenously, they may have potential to affect the cardial and cerebral function [19]. Despite those limitations, modification and innovation have been extensively developed in order to optimise the NPs formulations.

1.2.3 Potential pharmaceutical applications of NPs

NPs can be used to deliver various drugs, such as hydrophilic drugs [20], hydrophobic drugs, peptides, and vaccines [21] in prolonged period time of circulation. They can also be targeted carriers in the lymphatic system, brain, arterial walls, lungs, liver, and spleen [12].

Gene therapy

NPs have been investigated in clinical phase I of cysctic fibrosis therapy [22]. This investigation aimed to provide a single molecule gene of the cystic fibrosis transmembrane regulator (CTFR) in ultra small NPs (less than 25 nm) to slip through the nuclear membrane. The use of poly-L-lysine was reported to be in

association with reduced size, enabling internalisation of the gene to eventually reach the nucleus.

Ocular therapy

In ocular therapy, there have been several investigations involving NPs. Pilocarpine NPs has been reported as the first NP formulation in ocular delivery [23]. Polymethylmetachrylate was employed to prepare the NPs. This study demonstrated that Piloplex system decreased the intra-ocular pressure in clinical trials.

The efficacy of cyclosporin in nanocapsules was investigated by Calvo et al [24] as a comparison with oily solution cyclosporine. They revealed that there was corneal level induction five times higher than control. Campos [25] demonstrated the potential NP delivery of cyclosporine A in the eye using chitosan.

Cancer chemotherapy

In cancer chemotherapy, NPs were reported as promising carriers for anticancer agent such as doxorubicine [26], paclitaxel [27-29], peptides, and antiangiogenic genes.

Mu et al [30] demonstrated the manufacturing of PLGA nanospheres to deliver paclitaxel, a potent anticancer agent. Due to the poor solubility of paclitaxel, the formulation of this drug needs to be improved. In this study, Mu et al [30] also introduced the use of vitamin E TPGS as the stabiliser. Since vitamin E TPGS is natural and non toxic, no stabiliser removal was needed. This feature can be potentially valuable for NP formulation.

Research has been carried out in the development of antiangiogenesis therapy using NPs [22, 31]. This therapy aims to interrupt the blood supply for the tumors by diminishing the blood vessels around the tumors. However toxicity issues increase as the agent may not be selective to the tumor blood vessels; but may affect the normal vessels. NPs are thought to be a promising targeting delivery system to address this issue, since they can couple the antiangiogenic ligand, and, due to their small size, the NPs will be able to deliver the agent precisely and specifically into local endothelial cells [32].

Vaccine delivery

Singh et al. [33] demonstrated the manufacture of poly-(ε-caprolactone) (PCL), a poly(lactide-co-glycolide) (PLGA)-PCL blend and co-polymer NPs encapsulating diphtheria toxoid (DT) for a mucosal vaccine delivery system. Significantly higher uptake of PCL NPs was observed in an *in vitro* experiment using Caco-2 cells in comparison to polymeric PLGA, the PLGA-PCL blend and co-polymer NPs. Positive correlation between hydrophobicity of the NPs and the immune response was also observed following intramuscular administration and after intranasal administration of the NPs.

Immunopotentiation effect at modest doses of a few micro- or nanograms of CpG oligodeoxynucleotide (CpG ODN) and the influence on T cell responses at such low doses has also been investigated to establish NP delivery of vaccine adjuvant of tetanus toxoid. This study emphasises that antigen delivery in biodegradable NPs can facilitate the induction of strong T cell responses, particularly of the Th1 type, at extremely low doses of CpG ODN since NPs have an immunopotentiation effect on T cell response. This dose modification would be beneficial for minimising the potential side effects of these novel adjuvants [34].

1.2.4 Polymers used for NPs manufacturing

A large variety of polymers can be employed as matrix materials of the NPs to provide a controlled release effect. They are categorised as biodegradable and non-biodegradable polymers. Since the current study involves biodegradable polymers, which have potential value in a controlled drug delivery, this review will mainly focus on those polymers.

Biodegradable polymer is a polymer which can be metabolised either enzymatically or non enzymatically *in vivo*, to produce biocompatible by-products, which then can be eliminated by normal physiological pathways [35]. Biodegradable polymers have shown several advantages over non-biodegradable polymers. Once they were implanted, the biodegradable polymers do not need surgical removal from the body. They are degraded over time in a controlled decay, in which the side effects on the tissues can also be decreased [36]. However, biodegradable polymers also have a

limitation as they are difficult to remove in the case of therapy rearrangement. In terms of safety, monitoring of the reaction affected by the degradation products must also be carried out [3, 37].

In addition to biodegradability, other properties of polymer such as crystallinity and glass transition temperature are also important considerations in selection of a polymer for NP formulation. In terms of crystallinity and glass transition temperature (T_g), polymers can be divided as glassy polymers (T_g > 37ºC) and rubbery polymers (Tg < 37ºC). The glass transition temperature is associated with the permeability of the polymers. The permeability will increase with the decrease of the T_g. To achieve sufficient permeability, an approach to decrease the T_g can be applied by the addition of methylene groups to the backbone of the polymer structure, as well as introducing an asymmetric center. Increasing the crystallinity as well as T_g, however, can be carried out by the addition of aromatic groups to the polymer backbone. This manipulation aims to increase the mechanical strength of the polymer since the ideal matrix should be soft and pliable (Tg < 37ºC) but should maintain the tensile strength afterwards [3]. Chawla et al [38] demonstrated that microchannel structures are formed in a highly crystalline matrix, resulting in a high effective area for drug diffusion.

Natural hydrophilic polymers

Natural hydrophilic polymers have been reported to be potential matrices for encapsulating the drug in NPs. They are divided into two major categories: proteins (albumin and gelatine) and polysaccharides (alginate, dextran, chitosan).

Chitosan, as an example of natural hydrophilic polymers, is synthesised from chitin deacetylation [25, 39-41]. It has been widely used as a carrier in controlled delivery systems. Chitosan is a cationic polymer and due to a strong positive surface charge, it can be well adhered with a negatively charged surface and chelates metal ions [42]. This polymer may potentially be applied for developing the delivery of cyclosporine A to the cornea and conjuctiva, due to its ability to remain attached onto the surface for at least 24 hours [25] and also development of vaccines delivery [40, 41].

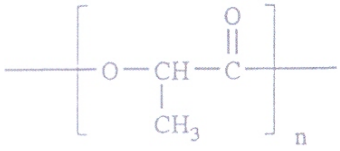
However, natural polymers have been reported to have less capability to provide the range of ideal characteristics to be such carriers in a controlled release study, since they may degrade the drug embedded because of required cross-linking, they are sensitive to microbial growth and enzymatic degradation [12] and they may induce immune response [43]. Therefore, synthetic hydrophobic polymers have been extensively investigated as alternative drug carriers in the manufacturing of NPs.

Synthetic hydrophobic polymers

Synthetic hydrophobic polymers have attracted much attention in developing the nanoparticle (NP) formulation. They offer advantages in that they are biocompatible and can be relatively good carriers without showing undesirable interaction with the drug. Although they degrade over time, there is no report of microbial or enzymatic activities involved in the process of degradation. Of those polymers, only poly (D,L,-lactide) and poly (D,L,-lactide-co-glycolide) (PLGA) will be reviewed, since those polymers were used in the current study. Those polymers have been widely investigated as drug carriers since they are biodegradable and biocompatible therefore they have regulatory approval in most countries. Toxicological and clinical data of those polymers is readily obtainable. Moreover, those polymers are commercially available [43, 44].

Poly (D,L,-lactide) (PDLLA)

PDLLA is completely amorphous with a glass transition temperature of around 57ºC. Since it is more amorphous than the other isomer, poly (L-lactide) (PLLA), the tensile strength as well as the modulus of elasticity of this polymer are lower, ~5000 psi and ~250,000 psi respectively. The consequence of this characteristic is that this polymer will be degraded faster than PLLA [45]. The group of lactic acid indicates that the poly lactic acid is hydrophobic (Figure 1.2) with a level of hydrophobicity greater than Poly(D,L,lactide-co-glycolide) but less than poly(L, lactide).



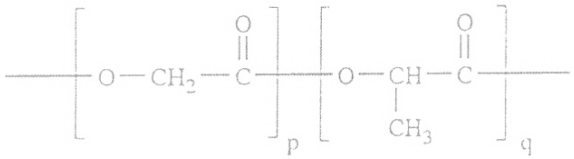
Poly(lactic acid) or PLA

Figure 1.2: Structure of poly (lactic acid)[43]. The letter n represents the number of lactic acid monomers

Poly (D,L,-lactide-co-glycolide) (PLGA)

PLGA has been clinically approved by the FDA as a biodegradable and non-toxic polymer[46]. This polymer was initially applied as a suture, which did not require surgical removal on its application. Its advantages, such as excellent biodegradability, biocompatibility, mechanical strength, hydrophobicity and flexibility, make this the most commonly used polymer in a controlled delivery system.

PLGA is synthesised by polymerisation lactide/glycolide in various ratios. PLGA is copolymer of poly(lactide) (PLA) and poly(glycolide) (PGA), which can be well-absorbed into the body when it is degraded. The chemical structure of PLGA can be seen in Figure 1.3. Gurny[15] suggested that the rate of PLGA biodegradation increased with increasing the glycolic unit proportion in this polymer.



Poly(lactic acid-*co*-glycolic acid)

Figure 1.3: Structure of poly (D,L, lactide-co-glycolide) [43]. The letter p represents the number of glycolic acid monomer, q represents the number of lactic acid monomer

1.2.5 Synthesis of NPs

A variety of methods have been developed to synthesise NPs. Those methods can be classified based on the performed matrix polymers, whether they are from the monomer which undergoes polymerisation or directly from the macromolecules. In this review, the most important methods of NPs preparation are described.

Coacervation/phase separation

Coacervation/phase separation techniques can be separated into two categories: aqueous phase separation and organic phase separation. The basic principle of this method is the addition of desolvating agent which can decrease the solubility of the matrix, leading to phase separation. In this separation, polymer and coacervates exist in one phase, and free-polymer-supernatant is in the other phase.

Solvent diffusion (nanoprecipitation or solvent displacement) method

The solvent diffusion method has been very popular in the preparation of NPs in recent years. Chorny [47] reported that this method can produce NPs sized less than 100 nm with a narrow distribution using several modifications. This is suitable to the preparation of highly-intracellular uptake-NPs. Another advantage of this method is that the method requires relatively non-toxic organic solvents, such as acetone, or a mixture of acetone and ethanol; therefore the risks of potentially toxic solvents can be avoided [47-49]. High energy output equipment can also be avoided using this method, leading to the possibility for this method to be scaled up [50].

The principle of this method is a precipitation of polymer or drug with polymer in the form of NPs as a result of rapid diffusion of one or two miscible organic solvents, in which polymer or a combination of polymer and drug, were dissolved into water. The basic procedure was a dropwise addition of the polymeric organic solution into an aqueous solution containing a stabiliser and followed by continuous stirring at room temperature to reach equilibrium.

Murakami et al.[48] established a modified spontaneous emulsification solvent diffusion method. In this development, they suggested that a proper composition of

binary organic solvents (ethanol and acetone) and the polymer concentration can present NPs with better characteristics, such as submicron particle size and higher yield efficiency (Figure 1.4). In their experiment, ethanol added to a PLGA acetonic solution, was also shown to inhibit the aggregation.

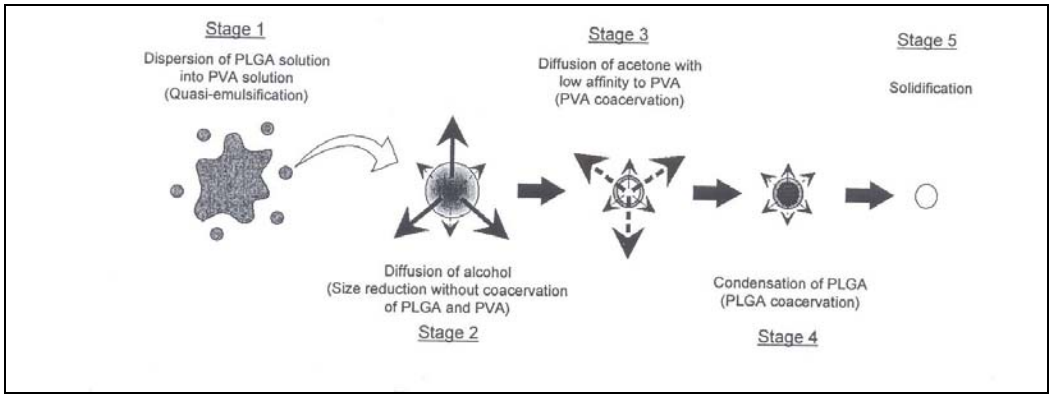


Figure 1.4: Solvent diffusion process illustrated by Murakami [48]

The mechanism involved in the solvent diffusion method can be explained by a study reported by Murakami et al [48, 49]. In their study of developing a modified spontaneous emulsion solvent diffusion, they introduced a mixture of two organic solvents, which were ethanol and acetone. They demonstrated that there was a perturbation of the interface during the dispersion of PLGA solution into the aqueous PVA solution, leading to the spontaneous formation of a large interfacial area. This phenomenon was governed by the so called Marangoni effect, resulting in nano-sized quasi emulsion droplets of PLGA solution. The ethanol initially diffuses out from the droplet due to the lower affinity of ethanol to PLGA, followed by acetone. Acetone subsequently diffused, resulting in PVA coacervation in the aqueous phase. This diffusion allowed an increased concentration of PLGA, leading to the solidification or precipitation of PLGA and PVA adsorption simultaneously. This spontaneous and instantaneous process allowed the formation of uniform NP dispersion under mild agitation.

Regardless of the advantages described above, several disadvantages have also been uncovered, such as less drug load due to leakage (especially for hydrophilic drugs) or

crystallisation of the drug (for hydrophobic drugs) during the process, and less yield due to initial aggregation of the polymer and the physical instability of the NPs.

Emulsification-solvent evaporation

This method involves organic solvents which are immiscible with water. Emulsification-solvent evaporation has been successful in encapsulating the drug to produce NPs as well as microparticles, of either hydrophilic or hydrophobic drugs. The ability to load higher amounts of the drug, especially the hydrophobic drugs or drugs with poor solubility, indicates this method is superior to the solvent diffusion method. However, the use of potentially toxic solvents should be taken into consideration. The appropriate solvent removal method should be carried out following this method of NP preparation.

The mechanism of solvent evaporation is different from solvent diffusion. The reduction of particle size is not governed by organic solvent diffusion, but by a vigorous agitation in the formation of primary emulsion droplets. The organic solvent of this method is subsequently removed by evaporation after a formation of emulsion droplets [12]. Increasing the energy of agitation results in the smaller particles. To achieve nano-sized particles, therefore, the aid of sonication is needed.

This method does not only allow the hydrophobic drugs to be encapsulated, but also hydrophilic drugs, by employing water - in oil - in water (W/O/W) emulsion [51]. Basically, the hydrophilic drug is dissolved in the aqueous phase, then it is emulsified with an organic solvent containing polymer. This emulsion is then emulsified with aqueous phase containing the stabiliser. By this process, the hydrophilic drug should be well encapsulated in a polymer matrix. The oily phase will prevent the drug to diffuse to the external aquoeus phase. Solidified NPs are then produced by removing the organic solvent and it is followed by the drying procedure to collect the dry, solid NPs.

1.2.6 Surface modification of NPs

Conventional hydrophobic NPs in blood circulation may be prone to the rapid clearing by opsonisation and macrophages. Therefore, considerable effort has been

made in order to prevent the NPs from early clearing and to prolong the circulation in the blood. It is suggested that to prevent phagocytosis, particles should be larger than 3 μm [3]. However, this suggestion apparently does not work in NP development.

Surface modification has been developed to tailor the problem [9]. Coating the surface with hydrophilic polymers allows the hydrophobic NPs to be retained longer in the circulation due to steric stability offered by hydrophobic polymer. This approach may also control the opsonisation and improve the surface properties of the NPs. Among those surface coated polymers, PEGylated-poly lactide (methoxy poly lactide mPEG PLA) and PEGylated PLGA(methoxy-poly lactide-co-glyclide, mPEG PLGA) have gained much interest in the NP investigation. Their basic colloidal properties and degradation depend on copolymer composition. The mPEG PLA and mPEG PLGA NPs exhibit prolonged blood circulation following intravenous administration to animals. The composition of NPs determines their biodistribution properties, probably through its influence on the effectiveness of the PEG steric barrier and the size of the NPs. The ability of the mPEG PLA and mPEG PLGA NPs to avoid rapid phagocytosis has extended the range of sites within the body that the NPs can reach, which has significant implications with regard to their application in controlled drug delivery and targeting. The mPEG PLA and mPEG PLGA NPs can be loaded with a variety of bioactive agents achieving satisfactory loading, especially in the case of hydrophobic drugs. The NPs have been investigated for the treatment of infectious diseases and cancer, the intravenous and mucosal delivery of proteins, and oligonucleotide and gene delivery [52-54]. This current study is investigating mPEG PLGA as one of the polymers used in nanoparticle formulations. The chemical structure of mPEG PLGA is shown in Figure 1.5.

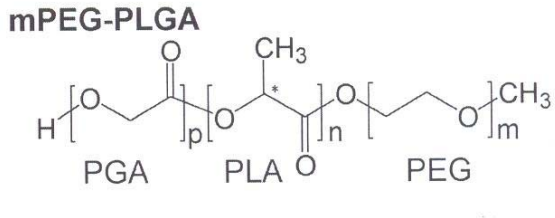


Figure 1.5: Structure of (methoxypolyethyleneglycol)poly-(D,L,lactide-co-gycolide)[13]

1.2.7 Physical characterisations of NPs

To characterise the NPs, there are mainly five parameters to be taken into consideration: particle size and morphology of particles, surface charge, drug loading and release study.

One of the most important features of NPs is particle size. It has been a challenge in NP formulation to measure the particle size accurately in the presence of dust, microbial contamination and crystallisation or aggregation of the ingredients that occur during the synthesis. However, photon correlation spectroscopy or dynamic light scattering is the currently applicable method to measure the size of sub-micron particles. It measures the hydrodynamic diameter which is a combination of the particle diameter and the double layer thickness. This method offers several advantages: it enables small and non-invasive sampling, automatic measurement, and is less time-consuming with high accuracy results. The basic instrumentation of this method is a coupling between low-angle laser light scattering and a detector in which the scattered waves travel to the detector. It is suitable to measure the size of particles which undergo a diffusive Brownian motion. Nevertheless, there are several drawbacks to be taken into consideration. This method has a limitation of measurement up to 3μm. Larger particles are subject to laser diffraction [55]. The measurement of particle size is normally verified by the examination of the surface morphology of NPs. Scanning electron microscopy enables the researcher to investigate the surface morphology of the NPs.

Various parameters were reported to highly affect the particle size in NPs preparation. Polymer concentration, internal/external ratio, exchange ratio and solvent-polymer interaction, as well as stabiliser type and concentration are several important parameters in optimising the NPs size. Increasing polymer concentration of organic phase added into a stabiliser solution results in poor dispersability of the organic phase due to higher viscous resistance against shear forces during emulsification. This will produce larger and heterogeneous droplets, which eventually result in larger particles [56, 57]. The increased internal/external (organic phase/ aqueous phase) ratio may prevent coalescence of droplet due to the larger amount of solvent in the organic phase, resulting in reduced size of particles [56].

Choi et al [58] demonstrated that if the exchange ratio, the ratio of diffusion from solvent to water over diffusion from water to solvent, is increased and the solvent-polymer interaction parameter is decreased, a small supersaturating region will be created, resulting in small NPs. Stabiliser type and concentration also affect the size of particles in terms of the ability to stabilise the emulsion during preparation. Generally, higher stabiliser concentration is more likely to produce smaller particles. However, Sahoo et al [59] suggested that for PVA, the concentration of stabiliser used in the NPs preparation should not be higher than 2.5% since PVA is aggregated at that concentration and tends to show surfactant activity, leading to the formation of larger particles after solvent evaporation. Since those parameters were fixed in this study, the difference properties of TA-NPs were mainly because of the nature of the polymers.

Surface charge, which can be determined by measuring the zeta potential, is an indicator for stability of the NPs in the suspension. As the charge increases, the repulsive interactions will be greater to maintain physical stability of a suspension. The zeta potential can be measured by laser Doppler anemometry. In addition to determining the physical stability of NPs in the suspension, surface charge determines the acceptability of the NPs when they are administered in the body, regarding the phagocytic uptake of NPs by cells of a reticuloendothelial system [8]. A value of ± 30 mV is considered as the minimum value of zeta potential to provide a physically stable nanosuspension [12]. In terms of cellular uptake, negatively charged NPs show poor endocytosis while positively charged NPs exhibit rapid internalisation and accumulate at high extent [60]

Determination of the loading of NPs can be carried out in a direct or an indirect way. Direct determination, on the one hand, is carried out especially for hydrophobic drugs, which can be extracted from NPs. On the other hand, indirect determination is commonly used to determine the hydrophilic drug. In this technique, free drug in the supernatant is determined and is used to subtract the initial drug added to achieve the drug loading in the NPs.

The release characteristics of a drug is one of the major features in NPs due to their characteristics as a controlled delivery system. The profile of drug release is

markedly affected by several factors, such as physicochemical properties of the drug and the polymer, size of the particles, distribution of the drug incorporated into NPs, stability and the degradation rate of the polymer, the diffusion coefficient and affinity of the drug-polymer matrix[8, 61]. The drug can be released from the matrix by desorption of surface bound drug, diffusion through the matrix or polymer walls, erosion of the NPs or a combination of erosion of the matrix and diffusion of drug [8].

1.2.8 Purification of NPs

NPs produced by the available methods may contain contaminants and not be of acceptable purity. Poly(vinyl alcohol) (PVA) solution, which acts as a stabiliser solution when is used in high concentration, is potentially toxic and unacceptable for vascular administration. Excessive amounts of remaining organic solvent are likely to be harmful and potentially carcinogenic. Therefore, removal of those elements is important in the manufacture of NP system.

A variety of methods have been investigated for NP purification, including dialysis centrifugal device (DCD), tangential flow filtration (TFF) and high-speed centrifugation [62]. Among those methods, high-speed centrifugation is probably the most effective and simple way to collect the NPs and remove the impurities. This method is reported to able to remove approximately 90% of PVA from the system [62]. Although the high centrifugal forces may cause a compaction of NPs, especially for the hydrophobic matrix, the compaction still can be redispersed in a reasonable manner.

1.3 *In vitro* cell culture toxicity study

Cell culture has been widely applied to facilitate *in vitro* toxicity studies. According to Freshney [63], cell culture refers to "cultures derived from dispersed cells taken from the original tissue, from a primary culture, or from a cell line or cell strain, by enzymatic, mechanical, or chemical disaggregation." Cell culture is also commonly employed for investigating various aspect of cell biology and physiology [64].

1.3.1 Significance of cell culture for *in vitro* study

Cell culture offers an advantage of precise control of physicochemical environment and physiological conditions, which is always kept constant. It is also virtually identical and the results are more reproducible compared to *in vitro* study with excised animal tissue, therefore it minimises the use of statistical analysis of variance. In terms of economical reasons, cell culture requires less reagent and reduce the use of animals than *in vivo* study, therefore the study can be more cost effective. In addition, since cell culture only involve either animal or human cells, the ethical issue can be avoided [63, 64].

1.3.2 Assay used for *in vitro* toxicity studies

A variety of assays have been extensively applied for in vitro toxicity study using cell cultures. Those are categorised into two major classes: (1) a short-term response using a measure of viability such as membrane integrity or loss of metabolic function, during exposure to potentially toxic materials, (2) a long-term response of survival, such as expressing metabolic capacity after exposure.

The cell viability can be referred to membrane integrity of cells after being exposed to potentially toxic substances, which is determined by either dye exclusion or dye uptake. Dye exclusion refers to the concept in which viable cells are impermeable to several types of dyes, while dye uptake refers to the ability of viable cells to take up certain chemicals.

1.3.3 MTT assay

The MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was initially developed by Mosmann in 1983 [65]. It involves a reduction of yellow tetrazolium salt (MTT), to form insoluble purple formazan crystals. The cleavage of tetrazolium salt to yield insoluble formazan product, which then are accumulated within cells, can only be carried out by the mitochondrial dehydrogenase enzyme, produced by the active mitochondria of living cells. The purple formazan product can be solubilised by detergent, dimethyl sulfoxide (DMSO) or isopropanol and quantified colorimetrically.

MTT assay is proven as a rapid and convenient assay to determine the cellular growth and cell survival. It requires a short period of time of analysis and a simple spectrophotometry. Moreover, this method offers high sensitivity and accuracy, since it can detect slight changes in cell metabolism. The linearity between cell number and absorbance, which can be well established, shows the accurate and direct quantification of viable cells and proliferation. This assay is also considered to be safer than the other methods, since it does not require radioactive substances [65]. Moreover, this assay is widely acceptable in toxicity studies, especially for NP formulations, with good correlation with *in vivo* toxicity.

1.3.4 Cell lines as the objects for the current study

The BALB/c 3T3 cell lines are continuous, immortalised but not transformed cell lines which are not tumorigenic in mice [66]. They are reported as the cell model used in the in vitro toxicity evaluation of genistein glycosides using neutral red uptake (NRU) assay [67].

The ARPE 19 cell lines are continuous immortalised cell lines established from human retinal epithelial cells. It is derived in 1986 by Amy Aotaki Keen [68] from the normal eyes of a 19-year old male who died in a motor vehicle accident. Dunn et al [68] demonstrated that ARPE 19 cells showed a rapid growth by passage 5, forming cobblestone monolayers, which pigmented after several months. They demonstrated that ARPE 19 has structural and functional properties characterisation of RPE cells in vivo, therefore this cell line is invaluable for in vitro studies of retinal pigment epithelia.

1.4 Triamcinolone acetonide (TA)

Triamcinolone was first introduced in 1958 as a potent anti-inflammatory agent. Its antirheumatic activity was reported as similar to methylprednisolone, but greater than prednisolone [69]. TA (9α-Fluoro-11ß, 16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-acetal with acetone) was developed as a derivative of triamcinolone. It has a molecular weight of 434.5 [70].

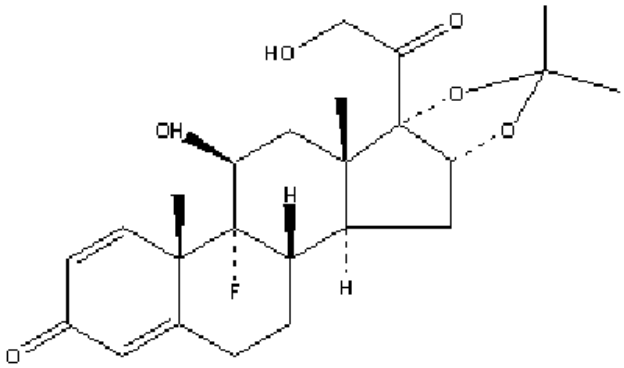


Figure 1.6: Structure of triamcinolone acetonide [71]

The 9α-Fluoro group attaches to the steroid ring (Figure 1.6) and governs the increase of the anti- inflammatory activity while the insertion of a 16α-hydroxy analogue is reported to minimise the mineralocorticoid activity [69].

1.4.1 Physicochemical properties

TA is chemically prepared crystalline powder with white, or almost white, colour in a solid state, with no distinct melting points. Several reports state different melting points of TA: 277 ºC, 292 ºC, and 294 ºC [72]. The difference is likely due to the different polymorph existing for the compound. TA is very slightly soluble in water, but soluble in ethanol (1:150), acetone (1:11) and chloroform (1:40). It is very soluble in dehydrated alcohol and methanol but sparingly soluble in ethyl acetate[73] .

TA is reported to be stable in its solid state. However, Gupta [74] demonstrated that aqueous-alkaline and ethanolic solutions of TA are unstable. The degradation of TA alkaline and ethanolic solution, which follows pseudo-first order kinetics, is governed by oxidative rearrangement of the α-ketol side chain. He also showed that TA solution is more stable at pH values less than 5.5 and minimal degradation occurred at pH 3.4. However, it was demonstrated that, above pH 7, the decomposition decreased with an increase of the ionic strength. In addition, the solution stored at 25ºC was nine times more stable that of at 50ºC. Another study, which was conducted by Timmins and Gray [75], also demonstrated that TA follows specific acid catalysis at pH 1 to 3, and specific base catalysis at pH 4 to 7 an pH 9 to 12, with the pH independent region at pH 7-9. The pKa of this drug is only stated in

terms of TA phosphate (1.70) [70]. As TA does not ionise, no information on the pKa of TA is available.

1.4.2 Clinical applications

As a corticosteroid, TA is known to treat various diseases related to autoimmune and allergic conditions. It was first introduced in topical administration, which showed better efficacy than other corticosteroids. The effect of TA is mainly related to its glucocorticoid action and suppression of inflammatory responses. TA is a drug of choice to treat diseases associated with inflammation such as arthritis, given by intra articular injection to relieve pain. TA has also been reported to reduce lower back and radicular pain. In this treatment TA is administered via epidural injections of a microcrystalline TA suspension [69, 71, 76, 77 78].

Recently, TA has been extensively investigated in the treatment of a variety of ocular diseases associated with inflammation and neovascularisation [82, 84-90]. In its application for ophthalmic disease management, TA is a proven anti inflammatory agent and an inhibitor of neovascularisation [79]. TA is a drug of choice for treatment of symphatetic ophtalmia and post cataract surgery inflammation[77]. TA has also been indicated for other ophthalmic diseases such as age-related macular degeneration (AMD), diabetic macular edema and foveal telangiectasia[82, 83]. Its antiangiogenic effect is mediated through the mechanism of either alteration of extracellular matrix degradation or inhibition of angiogenic growth factors [79-81].
The angiostatic effect of TA in treating neovascularisation [90, 91], therefore provides potential for this agent to be developed as an antiangiogenesis agent, which could be useful in the treatment of a range of cancers.

1.4.3 Side effects

TA may suppress the normal inflammatory responses which may activate the latent infection caused by tubercular or fungal. The use of large amount of TA can lead to the failure of producing adrenal suppression, diabetes, hypertension and Cushing's syndrome. Prolonged used of TA may develop Chusingoid body changes and osteoporosis which is irreversible[71]. Those side effects are related to high dose chronic systemic administration.

In ocular therapy, TA in suspension is commonly administered to patients with AMD or other retinal diseases via intra vitreal and sub-tenon's injection [87, 88, 92]. However, there are several side effects associated with those treatments, such as increasing the intraocular pressure (IOP) and the risk of post injection infectious endophthalmitis.

TA is commercially available as a cream or gel for topical administration and a microsuspension for parenteral administration. In ocular therapy, a suspension of TA is commonly administered to a patient with retinal diseases via intra vitreal and sub tenon's injection [82, 88, 93, 94]. However, there are several side effects associated with those treatments, such as: increasing the intraocular pressure (IOP) and the risk of post injection infectious endophtalmitis [92, 95]. TA itself is reported as a steroid-induced risk of ocular hypertension and cataract development regardless of the dosage or particle size [77, 92]. Other side effects reported are immune system depletion, moon face, and osteoporosis [77], associated with high chronic oral doses of corticosteroid. Having those potential side effects, the administration of TA should be improved to minimize the side effects.

1.5 Objectives of the study

Over the years, the research focused in our laboratory has been to develop NP formulation for delivery of various drugs and peptides.

TA is known as potential anti inflammatory agent and has been extensively investigated in the treatment of retinal diseases, such as age related degeneration, diabetic macular oedema and neovascularisation [79-83]. The ability of TA to inhibit neovascularisation can be proposed as a potential indication for TA to be an antiangiogenic agent in the cancer chemotherapy.

TA is commercially manufactured as a microsuspension for parenteral administration. Evidence suggests that TA can potentially induce toxicity in that form at typical dosages [92]. Despite this, there has only been very limited effort directed towards to TA formulation to achieve a safer and more acceptable means of delivery. Although the delivery of TA by NPs has been investigated by Krause [96],

there is a need for further research of TA loaded NPs, using other biodegradable polymers and potentially superior methods, to improve the quality of NPs.

The overall aims of this study were to develop TA loaded-NP formulations from biodegradable and biocompatible polymers, which provide: sustained release, prolonged stability and low toxicity.

In order to achieve the overall aims, the following studies were set up to accomplished:

- Formulation of TA loaded NPs (TA-NPs) using poly(D,L,lactide) (PDLLA), poly(D,L, lactide-co-glycolide) (PLGA) and methoxy-poly-ethylene glycol-poly(D,L, lactide-co-glycolide) (mPEG-PLGA),
- Determination of the physical properties of empty NPs and TA-NPs,
- Investigation of the effect of NP incorporation on the stability of TA,
- Investigation of the effect of polymer on the *in vitro* release characteristics of three different types of TA-NPs,
- Assessment of the toxicity profiles of both empty NPs and TA-NPs in two *in vitro* cell culture models: BALB/c 3T3 and ARPE 19.

MATERIALS AND METHODS

2.1 Experimental materials

2.1.1 Experimental materials for TA-NPs formulation

- Acetonitrile; HPLC grade (LabScan, Thailand);
- Acetone; HPLC grade (LabScan, Thailand);
- Boric acid; analytical grade (Ajax Chemical Ltd, Australia)
- Dichloromethane; HPLC grade (LabScan, Thailand)
- Di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) 'AnalaR'; analytical grade (Merck Pty.Ltd., Victoria)
- D,L-Lactide/Glycolide copolymer (PLGA), ratio 85:15, batch number: 82/136, (Purac Biochem bv, Netherlands)
- Orthophosphoric acid, Mw 98.00, BDH 'AnalaR'; analytical grade, Min 85% (BDH, Germany)
- Poly (D,L,-lactic acid) (PDLLA), lot number: DG 676 GA, (Purac Biochem bv, Netherlands)
- Poly(lactic-co-glycolic)-methoxypoly(ethyleneglycol) (mPEG-PLGA) synthesized in house, containing 4.8% mPEG,
- Poly(vinyl alcohol) (PVA), 87-90% hydrolysed, Mw 30,000-70,000, batch number 094K0104, chemical grade (Sigma Aldrich Inc., USA)
- Sodium azide, (NaN_3) batch number: 1001010, analytical grade (BDH Chemicals Pty Ltd., Kilsyth, Victoria)
- Sodium chloride (NaCl), batch number: 55914, analytical grade (Scharlau Chemie, Spain)
- Sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$) 'AnalaR', analytical grade (Merck Pty.Ltd, Victoria)
- Triamcinolone acetonide (TA) micronised, batch number: 2196NMO0100421, chemical grade (Farmabios, Italy)
- Water (deionised) - passed through a milli 'Q' apparatus with an initial specifc conductivity of 18 MΩ cm at 25˚C (Millipore Corporation , Bedford, MASS, USA)

- All other chemicals were of analytical reagent grade.

2.1.2 Experimental materials for *in vitro* toxicity study

- ARPE 19 cell line, kindly donated by Adj. Assoc. Prof. Chooi-May Lai (Lions Eye Institute, Western Australia)
- BALB/c 3T3 cell line (ATCC, USA)
- Dimethyl sulfoxide (DMSO), analytical grade (Sigma-Aldrich Chemie, Germany)
- Media for culturing ARPE 19:
  Dulbecco's Modified Eagle's Media (DMEM) : Ham's F12 1:1 media (GIBCO, Invitrogen Co., USA) containing 10% fetal calf serum (JRH, Germany), 100 IU Penicillin-100 µg/ml Streptomycin (GIBCO, Invitrogen Co., USA),
- Media for culturing BALB/c 3T3 cells:
  DMEM containing sodium pyruvate (GIBCO), 10% fetal calf serum (JRH, Germany), 100 IU Penicillin-100 µg/ml Streptomycin (GIBCO, Invitrogen Co., USA) and 2 mM Glutamax (GIBCO, Invitrogen Co., USA)
- Methylene blue, analytical grade (Sigma-Aldrich Chemie, Germany)
- 0.9% NaCl / saline solution for irrigation (Baxter, Australia)
- Trypsin-EDTA (GIBCO, Invitrogen Co., USA)
- Triamcinolone acetonide (TA), micronised, (batch number: 2196NMO0100421 (Farmabios, Italy)

2.2 Instrumentation

2.2.1 Instrumentation for TA-NPs formulation

- Sonicator; Biosonik II (Bronwill Scientific, New York, USA),
- Bath sonicator; Bransonic Ultrasonic cleaner 2510E-DTH (Branson Ultrasonic Corporation, USA);
- Centrifuge; Allegra™ 64R centrifuge (Beckman Coulter, Inc., USA); Eppendorf minispin AG 22331 (Eppendorf, Germany),
- Field Emission Scanning Electrone Microscope (FESEM); Jeol JSM 6700F Japan, with a sputter coater (JFC-1300, Auto Fine coater, Jeol, Tokyo, Japan)

- High Performance Liquid Chromatograph (HPLC) apparatus, consists of:
- An Agilent 1100™ HPLC; an installation of a degasser (G1322A, serial # JP73022182), a quaternary pump (G1311A, serial # DE91610024), an automatic sampler (G1313A, serial # DE91612069), a diode array detector (G1315B, serial # DE01609022) and a ChemStation working station
- An Econosil™ column, C18 10 µm, 250 x 4.6 mm (Alltech Associates Inc, PA, USA), as the column for a reverse-phase HPLC analysis;
- NP sizer; Zetasizer 3000HS, Malvern Instrument, UK,
- Magnetic stirrer; Variomag telemodul 20P (Dr Hoss + Partner GmBH, Germany),
- Oven (Memmert, Germany);
- pH meter; Microprocessor pH/mV/ºC Meter, Model: 8417N, (Hanna Instruments, Singapore),
- UV-Vis spectrophotometer; diode array spectrophotometer 8452 A (Hewlett Packard, USA) and UV-Visible spectrophotometer 1201 (Shimadzu, Japan),
- Vacuum rotary evaporator; Buchi Rotavapor R-200 (Buchi Labortechnik AG, Switzerland).

2.2.2 Instrumentation for *in vitro* toxicity study

- Aseptic hood; Email™ Air Handling Hood, Biological Safety Cabinet, Type: Class II 1800 RH, conforming to 2252 part2 1994, Model No. 1687, (WestingHouse, Australia),
- CO_2 incubator; Sanyo CO_2 incubator, MCO-17AIC, (Sanyo Electric Co, Ltd, Japan),
- Centrifuge; Eppendorf™ centrifuge 5417C (Hamburg, Germany), Beckman GS-6 centrifuge, Germany
- Phase contrast microscope; Nikon phase contrast microscope, model TMS, Japan
- Microplate reader; Microplate reader model 3550 (BioRad, USA)
- Water bath, Thermoline™ water bath, Australia

2.3 Methods

2.3.1 High Performance Liquid Chromatography (HPLC) assay validation for TA

The TA HPLC method was adopted from a reported method [74] with some modification. An Econosil column, C18 10 µm, 250 x 4.6 mm (Alltech Associates Inc, PA, USA) was used as the stationary phase. The mobile phase consisted of a composition of acetonitrile (ACN) and 20mM phosphate buffer solution (pH 4.2) in the proportion of 50:50 v/v, filtered through a 0.45 µm membrane filter prior to use. The flow rate of 1.5 mL/min and the injection volume of 20µl were used. The assay validation was conducted with TA standards dissolved in two media: the ACN:mobile phase 1:1 v/v and phosphate buffer saline pH 7.4 containing 0.05% w/v sodium azide (PBS-NaN_3). To obtain a maximum sensitivity, TA was measured at its wavelength of maximum absorption: 242 nm. The temperature of the system was maintained at 25ºC. The HPLC assay of TA was validated for linearity, precision, recovery (accuracy), sensitivity and selectivity.

Linearity

Linearity of the assay was determined in two types of media for TA loading and release study i.e. ACN:mobile phase 1:1 v/v and PBS-NaN_3, respectively. The standard solutions were injected directly into the HPLC column under the conditions described in Section 2.3.1. The calibration curve was constructed for each type of medium by plotting peak areas against the concentration of TA standard solutions.

TA in ACN:mobile phase 1:1

Six standard solutions at concentrations of 1, 2, 5, 10, 30, and 40 µg/mL were prepared by diluting a 50 µg/mL of TA stock solution in ACN:mobile phase 1:1 v/v.

TA in PBS-NaN_3 pH 7.4

A 50 µg/mL of TA stock solution in PBS-NaN_3 was diluted to obtain standard solutions ranging between 0.5 µg/mL and 25 µg/mL. Due to the TA solubility problem, the stock solution was prepared by dissolving 12.5 mg TA in 100 mL of ACN and making up to 500 mL with PBS-Na N_3.

Preparation of PBS-NaN_3 solution

The method of preparation for Sorensen buffer was adapted from the Pharmaceutical Codex (ref) to prepare 40 mM phosphate buffer saline solution (PBS) pH 7.4, with a replacement of the di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$) with anhydrous di-sodium hydrogen orthophosphate (Na_2HPO_4) containing an equivalent amount of salt. The solution was prepared by dissolving 7.6 g of Na_2HPO_4, 2.1 g of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$), and 4.4 g of sodium chloride (NaCl) in 1000 mL of Milli 'Q'water. Sodium azide, NaN_3 (0.05% w/v) was included as a preservative in the PBS solution.

System precision

Six replicate injections of two different concentrations of TA (low and high concentrations) were assessed for the precision of the assay. The study was conducted in two media: loading medium and release medium. The means and relative standard deviations (RSD) were calculated for each TA concentration.

Recovery (accuracy)

Six concentrations ranging between 2.5 µg/mL and 40 µg/mL were analysed in the presence of a known volume of 20 µg/mL of empty PLGA, mPEG-PLGA (4.8%) and PDLLA NPs by the same method used for TA loading determination. The final solutions were filtered with a 0.2 µm syringe filter (GHP Acrodisc® 13, Pall Corporation, USA) prior to injection into the HPLC column. The recovery was calculated by comparing the actual TA concentration detected with the initial concentration.

Sensitivity

Blank solvents were injected six times consecutively into the HPLC column and the standard deviation of average noise levels in the expected retention time were determined. For the TA loading determination assay, blank solvent was defined as the loading media after being filtered from blank NPs, while in the release study, blank solvent was the PBS-NaN_3. The theoretical LOD and LOQ were then calculated by the following formulae:

$$LOD = \frac{standard\ deviation\ of\ noise\ levels}{slope\ of\ height\ versus\ concentration\ of\ calibration\ curve} \times 3 \qquad \text{(Eq.1)}$$

$$LOQ = \frac{standard\ deviation\ of\ noise\ levels}{slope\ of\ height\ versus\ concentration\ of\ calibration\ curve} \times 10 \qquad \text{(Eq.2)}$$

Selectivity

The peak purity of TA in two different media (ACN:mobile phase 1:1 and PBS-NaN_3) containing TA and degradation products were analysed to determine the selectivity of the assay. The interference of the media was also investigated. The purity factor should be within the calculated threshold limit.

2.3.2 Preparation of TA-NPs

Solvent diffusion method

The solvent diffusion method in this study was developed based on the method reported by Murakami[48].

Polymeric organic phase preparation

To prepare the polymeric organic phase, 0.4 g of polymer was dissolved in 20 mL acetone. The solution was then mixed well and served as the organic phase.

Aqueous phase preparation

Polyvinyl alcohol (PVA) with concentration of 0.6% (w/v) was used as the aqueous phase as well as the stabilizer solution. To prepare the PVA solution, 0.6 g of PVA was sprinkled onto 100 mL water and stirred overnight. The PVA solution was further filtered by a 0.2 μm syringe filter (GHP Acrodisc® 13, Pall Corporation, USA) to obtain a particle-free-solution.

Preparation of TA-NPs by solvent diffusion

Polymeric organic phase (20 ml) containing 80 mg of TA was added drop-by-drop into 60 mL of 0.6% PVA solution and constantly stirred at a speed of 300 rpm, at room temperature, for 4 hours to evaporate the acetone. The NP suspension was left on the bench for 30 minutes prior to purification. These procedures were repeated for

each type of polymer. The empty NPs were prepared with the same method without the drug.

Emulsification solvent evaporation method

In this method, 0.4 g of polymer was dissolved in dichloromethane (DCM). The solution was mixed well and prepared as the organic phase. The same stabilizer solution as per section 2.3.2 was also used in the emulsification-solvent evaporation.

Preparation of TA-NPs by solvent evaporation

Polymeric organic phase (20 mL) containing 80 mg of TA, and 40 mL of 0.6% PVA solution were sonicated using the Biosonic® (Bronwill Scientific, USA) for 2 minutes. The emulsion was then mixed with 20 mL of 0.6% PVA and continuously stirred for 10 minutes. The remaining DCM was removed by a rotary evaporator. The NP suspension was left on the bench for 30 minutes prior to purification. This procedures were repeated for each type of polymer. The empty NPs were prepared with the same method without the drug.

2.3.3 NPs purification

After evaporating the organic solvents, the NP suspension was centrifuged at 12,000 rpm for 20 minutes using an Allegra 64R centrifuge (Beckman Coulter, Inc., CA, USA). The supernatant was collected and used to analyse the amount of PVA removed from the NP suspension. The determination of PVA content was described below. The settled NPs were then dispersed in 10 mL of deionised water.

Determination of PVA content

A colorimetric method, reported by Dalwadi et al. [62], was adopted to determine the PVA content in the supernatant obtained during the purification of NPs.

To quantify the PVA in the nanoparticulate system, a calibration curve of absorbance versus PVA concentration was established. Standard solutions of PVA ranging from 18.9 µg/mL to 66.15 µg/mL were prepared from 2 mg/mL of the PVA stock solution. A volume of 5 mL of water was added into a known volume of standard solution, followed by the additions of 3 mL of 4% w/v boric acid and 0.6 mL of

0.1M iodine solution. The mixture was then made up to 10 mL with water accurately and kept in the dark for 5 minutes to optimize the formation of the complex. The PVA-iodine absorbance was measured using a UV-visible spectrophotometer 1201 (Shimadzu, Japan) at 690 nm.

Prior to PVA determinination, both supernatant obtained after NP centrifugation and PVA solution used to prepare NPs were diluted five times. To prepare the sample, 5 mL of water was added to 0.1 mL of diluted supernatant (and PVA solution), followed by the additions of 3 mL of 4% boric acid and 0.1M of iodine solution. After it was made up to 10 mL with water, the mixture was kept in the dark for 5 minutes. The absorbance was measured at 690 nm and the PVA concentration was quantified using a calibration curve prepared above. The PVA concentration was used to determine the PVA amount in the total volume of supernatant, which was equivalent to the amount of PVA removed from the nanoparticulate system. The percentage of PVA removal was calculated based on this equation:

$$PVA\ removed\ (\%) = \frac{amount\ of\ \ PVA\ in\ the\ supernatant}{amount\ of\ \ PVA\ in\ aquoeus\ \ phase} \times 100 \qquad \text{(Eq.3)}$$

2.3.4 NP characterization

Empty NPs and TA-NPs of each polymer were characterized in terms of morphology and size of NPs, zeta potential, yield, loading value, and entrapment efficiency.

Morphology and size of NPs

Morphology of the NPs was investigated using field emission scanning electron microscopy (FESEM). Briefly, the dried NPs were attached to the metallic studs with double sided carbon tapes and coated with gold by a sputter coater (JFC-1300, Auto Fine coater, Jeol, Tokyo, Japan) for 40 seconds with a 40 mA current intensity, prior to observation using FESEM (Jeol JSM 6700F, Japan).

The size of the NPs in diluted samples were measured using a Zetasizer 3000HS (Malvern Instrument Pty. Ltd., Worcestershire, UK) based on a dynamic light scattering (DLS) principle,. The hydrodynamic diameter, which is represented as Z

average mean size, was determined at 25º C with a detection angle of 90º. The triplicate measurements were performed at 25ºC.

Zeta potential

The surface charge of the NPs, expressed as zeta potential, was also determined by the Zetasizer™, with a zeta mode based on the laser droppler anemometry principle. The measurements were also performed three times with diluted samples at 25ºC.

Yield determination

A known volume of NP suspension was added to a pre-weighed glass container and placed into an oven at 70ºC until the dried NPs reached constant weight (evaporation of solvent complete). As a control, the same volume of water was also treated under the same condition. The yield of NPs was then determined by the following formulae:

Yield of blank NPs (Y_{NPs}):

$$Y_{NPs}\ (\%) = \frac{weight\ of\ \ dried\ \ nanoparticles}{weight\ of\ \ polymer} \times 100 \qquad \text{(Eq.4)}$$

Yield of TA-NPs ($Y_{TA\text{-}NPs}$):

$$Y_{TA-NPs}\ (\%) = \frac{weight\ of\ \ dried\ \ nanoparticles}{weight\ of\ \ (polymer + drug)} \times 100 \qquad \text{(Eq.5)}$$

Determination of drug loading and entrapment efficiency

A known volume of NP suspension was dissolved in 100 mL ACN to extract the drug from the NPs. The solution was then diluted 1:2 with the mobile phase, to precipitate the polymer. The precipitated polymer was removed by filtration with a 0.2 µm syringe filter (GHP Acrodisc® 13, Pall Corporation, USA) prior to injection onto the HPLC column. For analysis of TA concentration, drug loading was calculated based on the following formula:

$$Drug\ loading\ (\%) = \frac{amount\ of\ \ drug\ loaded}{weight\ of\ \ dried\ \ nanoparticles\ obtained} \times 100 \qquad \text{(Eq.6)}$$

Whereas the entrapment efficiency was determined by this formula:

$$Entrapment\ efficiency\ (\%) = \frac{amount\ of\ drug\ loaded}{amount\ of\ initial\ drug\ added} \times 100 \qquad \text{(Eq.7)}$$

2.3.5 Turbidity assay

Ten mL of 4 mg/mL TA in acetone was added into 30 mL of 0.6 % PVA and stirred. The samples were taken at time zero (t=0 min), t=2 minutes and at every 5 minutes thereafter and the mixture was continuously stirred. The absorbances of the samples were then determined using a diode array spectrophotometer 8452 A (Hewlett Packard, USA) at 400 nm. The sampling was carried out until the mixture reached equilibrium (the absorbance was relatively stable). The same method was used to study the precipitation of polymeric organic phase (20 mg/mL of PDLLA and mPEG PLGA) in acetone. The results compared the precipitation rate between the TA and polymers in the aqueous environment (0.6 % PVA solution).

2.3.6 *In vitro* release assay of TA-NPs

TA solubility study

TA was added into release media until over saturated. The suspension were stirred at room temperature under controlled agitation. The samples were taken over 8 hours and 24 hours and filtered by a 0.2 μm syringe filter (GHP Acrodisc® 13, Pall Corporation, USA) prior to injection onto the HPLC column.

Treatment of dialysis bag

The dialysis bags were washed with running water for 3-4 hours to remove the glycerine. Those bags were then immersed in 0.3% w/v of sodium sulphide at 80º C for 1 minute to remove the sulphur. The sodium sulphide was removed by washing the bags with water at 60ºC for 2 minutes and acidifying it with 0.2 % v/v sulfuric acid. The bags were finally rinsed with hot water to remove the acid and stored in Milli 'Q' water at 4º C. The water was replaced daily to minimize the risk of microbial contamination.

In vitro release method

The release study of TA-NPs was conducted using a dialysis bag diffusion technique. The experiment was performed in PBS-NaN_3 at 37º C. The equipment was set up as shown in Figure 2.1.

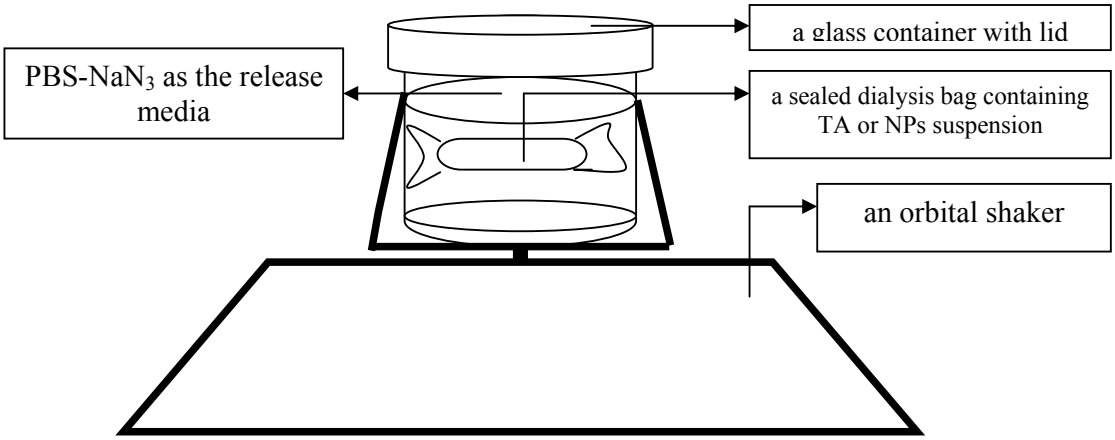


Figure 2.1: The dialysis bag diffusion equipment

In brief, 10mL of TA-NPs of each polymer (containing 2.5mg of TA) was added to a 4 cm of dialysis bag, which was then sealed. The sealed dialysis bag was then soaked in 50mL of PBS-NaN_3 and incubated at 37º C with constant shaking. Forty mL of samples were taken at 1, 2, 3, 4, 5, 6, 7, 8, 24, 72, and 96 hours, and replaced with an equal volume of fresh PBS-NaN_3 to maintain the sink condition. The TA concentration in the samples was then analysed using the HPLC assay under the same conditions as in section 2.3.1. The release study was conducted in triplicates for each type of TA-NPs.

Following controls were included in for the release study,

Control 1: TA suspension (containing 2.5mg TA in 10mL water) prepared by a similar technique of solvent evaporation for TA-NPs excluding the polymer

Control 2: TA suspension (containing 2.5mg TA in 10mL water) prepared by sonicating TA in water for about 5 min

2.3.7 Stability assay of TA

The stability study of TA in the release medium (PBS-NaN_3) was conducted under conditions with and without being loaded in the NPs.

Stability assay of TA in aqueous environment

The TA solutions with known concentrations were placed in 1.5 mL vials for sampling and kept under the experimental temperatures. Samples were taken at 3, 6, 9, 24, and 72 hours after initial incubation and determined by the HPLC assay as in section 2.3.1. The potency of TA was determined according to the following formulae:

$$Potency\ of\ TA\ (\%) = \frac{peak\ area\ of\ TA}{total\ peak\ areas\ of\ TA\ and\ degradation\ products} \times 100$$

(Eq.8)

The percentage of the degradation products was calculated by this formulae:

$$Degradation\ product\ (\%) = \frac{peak\ area\ of\ the\ degradation\ products}{total\ peak\ areas\ of\ TA\ and\ degradation\ products} \times 100$$

(Eq.9)

Stability of TA inside NPs

The stability of TA inside NPs was assessed after conducting the 96-hour *in vitro* release study of TA-NPs. The unreleased TA was extracted by the same method as described in section 2.3.4. The potency and the degradation products were determined using Eq.8 and Eq.9, respectively.

2.3.8 *In vitro* toxicity assay

The assay was carried out with two different cell lines, which are BALB/c 3T3 (fibroblasts) and ARPE 19 (retinal pigment epithelia) cell lines. The later was kindly donated by Adj. Assoc. Prof. Chooi May Lai (Lions Eye Institute), Western Australia. All related experiments were performed under aseptic conditions to avoid microbial contamination.

Cell culture

BALB/c 3T3 cells were maintained in Dulbecco's Modified Eagle Media (DMEM) containing 2 mM of L Glutamax®, and supplemented by 10% of fetal bovine serum (FBS), 100 U/mL of Penicillin, and 100 µg/mL of Streptomycin at 37ºC in humidified 5% CO_2 atmosphere. The media is then known with an initial of D10.

The ARPE 19 cells were maintained in 1:1 mixture of DMEM and Ham's F12 containing 3 mM of glutamine [97, 98] with the same supplementation and under the same conditions as of BALB/c 3T3, the media then is known as DF12.

Cell storage

For long term storage, cells were stored in cryovials in liquid nitrogen (N_2) storage. Prior to storage, 300 µl of FCS containing DMSO 9:1 (a freeze brew solution) was added into 300 µl the cell suspension in the cryovials as a cryoprotectant.

Thawing the cells

To bring up the cells from liquid nitrogen (N_2) storage, the cells were first thawed rapidly at 37ºC and then 5 ml of the appropriate media was added. The cell suspension was then centrifuged at 1000 rpm for 5 minutes, the supernatant was removed and the cells were resuspended in 8 mL of fresh media. The cells were then placed into 80 cm^3 flasks and incubated at 37º C.

Subculturing (passaging) the cells

Cell cultures were maintained until they reached approximately 80% confluence. The media was removed by aspiration, the cell monolayer was washed once with approximately 5 ml of saline and the cell harvested by addition of 1 ml of 0.2% of Trypsin-EDTA (Invitrogen, USA). Detachment of the cells was monitored by a phase contrast microscope. When the cells were detached, they were seeded into different flasks at an appropriate split ratio (1:3).

Cell counts

In order to standardize cell seeding into 96 well plates, cell counts were performed prior to seeding. Cells were harvested by Trypsin-EDTA as described above (in the section of subculturing the cells). Following detachment the cell suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and cells were resuspended in 2 ml of fresh media. To count cells, 20 µL of 0.4% Trypan blue was mixed with an equal volume of the cell suspension. The mixture was then added to the counting chamber and placed under the microscope. The cells were then counted on 25 squares with the following standard formula :

$$Amount\ of\ the\ cells/mL = (amount\ of\ the\ cells\ on\ 25\ squares) \times 2 \times 10^4 \quad \text{(Eq.10)}$$

Cell optimization

Cell optimization was conducted in order to determine the appropriate seeding density, which would provide an adequate response for the assay. The cell suspension (100 µL) was added in a 96-well plate in 3-4 different seeding densities (cells/well) and in three different plates, for three different examination times: 0, 24, and 48 hours. After 24 hour seeding at 37^{o}C, the procedure of the MTT assay, as described in section of toxicity assay below, was performed for the 0 hour-plate. The same procedure was then repeated with the 24 and 48 hour- plates after 48 hours and 72 hours seeding, respectively. The optimization graph of absorbance versus time for four seeding densities of the cells was constructed.

Toxicity assay

The MTT assay was performed on cells cultured in 96-well plates to determine the cell viability following 24 hour exposure to experimental samples. Cells were inoculated into 96 well plates in 100 µL of appropriate media at seeding densities of 15,000 cells/well for BALB/c 3T3 and 20,000 cells/well for ARPE 19. After cell inoculation, the cells were incubated for 24 hours prior to addition of experimental samples. After 24 hours, one plate was assayed to determine starting cell densities, this represent a measure of the cell population for each cell line at the time of samples addition (T_0). The cells were treated by addition of freshly prepared samples:

- TA in the media, in ten-fold concentrations ranging from 0.1 µg/mL to 1 mg/mL(four wells per concentration),
- TA-NP suspensions in media, of each polymer (PDLLA, PLGA, and 4.8% mPEG PLGA), containing various amount of TA as above, with various concentrations of NPs based on the loading value (four wells per certain amount of TA),
- empty NP suspension in media, of each polymer, containing an equivalent amount of NPs as used in the TA-NP suspesions above (four wells per certain amount of NPs).

Cells without any treatment other than an addition of 100 µL of media were used as controls. Following samples addition, the cells were incubated for an additional 24 hours and the assay is terminated by adding 100 µL of MTT solution (1 mg/mL in appropriate media). The cells combined with an MTT solution were then incubated for 1 hour at 37ºC. After the precipitation of purple fomazan, the MTT supernatant was removed and 100 µL of DMSO added to dissolve the purple formazan product. The absorbance of dissolved product was determined using a multiwell microplate reader 3550 (BioRad, USA) at a wavelength of 595 nm. The absorbance was corrected by reagents in the absence of cells and experimental samples. The toxicity assay was replicated three times. The toxicity of the samples is expressed as the percentage of cell viability in comparison to control cells. The control cells were assumed to be 100% viable.

2.3.9 Statistical analysis

All results of triplicate samples in each study are presented as mean ± SD, representing descriptive statistics. The statistical analysis for *in vitro* toxicity study was carried out using InStat® computer software. Unpaired t test was used to analyse the significance of the differences between TA and NPs. A significant difference was considered if the p value was less than 0.05 ($p<0.05$).

RESULTS AND DISCUSSION

This study was conducted with two main objectives. The first was to develop polymeric NP formulations for delivery of TA, which would provide: low toxicity, sustained release properties and prolonged stability of TA. The second objective was to assess the toxicity of TA loaded NPs (TA NPs) compared with TA alone upon exposure to BALB/c 3T3 (fibroblasts) and ARPE 19 (human retinal pigment epithelial) cell lines. Since conventional TA administration is known causing potential side effects, it is expected that the NPs developed for TA administration can minimize potential side effects of TA by providing a controlled drug delivery to a target site. There have been concerns on the toxicity of NPs themselves, therefore, it is essential to study the toxicity of NP formulations with and without incorporation of TA. The ultimate aim of this study was to develop a safe and acceptable polymeric NP formulation for TA delivery that could have potential in ocular delivery.

3.1 HPLC assay validation

To determine the TA concentration, a HPLC assay developed by Gupta [74] was adopted with some modification. It was found that the original mobile phase containing ACN:20 mM phosphate buffer pH 4.2 in the proportion of 32:68 with a flow rate of 3 mL/min could not produce a distinct peak of TA. In order to achieve an optimum HPLC assay, a modification was made to reduce the wavelength to 242 nm, change the proportion of mobile phase to ACN: 20 mM phosphate buffer pH 4.2 50:50 and alter the flow rate to 1.5 mL/min. The internal standard was excluded since the assay recovery was satisfactory with external standards.

The linearity of UV detector response was assessed for calibration curves in two different media: ACN:mobile phase 1:1 and PBS-NaN_3. The former was the medium for the analysis of TA loading within NPs, and the latter was the release medium in the *in vitro*-TA-release study. Sodium azide (NaN_3) was added as a preservative in the release media with the concentration of 0.05% to prevent microbial growth.

The range of TA concentration in the media of ACN:mobile phase 1:1 was from 1.03 µg/mL to 41.20 µg/mL, while in the release media, the concentration ranged from 0.264 µg/mL to 26.4 µg/mL. Correlation coefficient r^2, an indication of the fitness of linear regression, was calculated for the calibration curve of area under the curve (AUC) against the TA concentration in both media. The detector's response was found to be linear with r^2 of 0.9999 and 1 for ACN:mobile phase 1:1 media and PBS-NaN_3, respectively. This suggests that the concentration of TA in the samples can be determined against the standard in the concentration range of calibration curve. Representative calibration curves are presented in Figure 3.1 and 3.2.

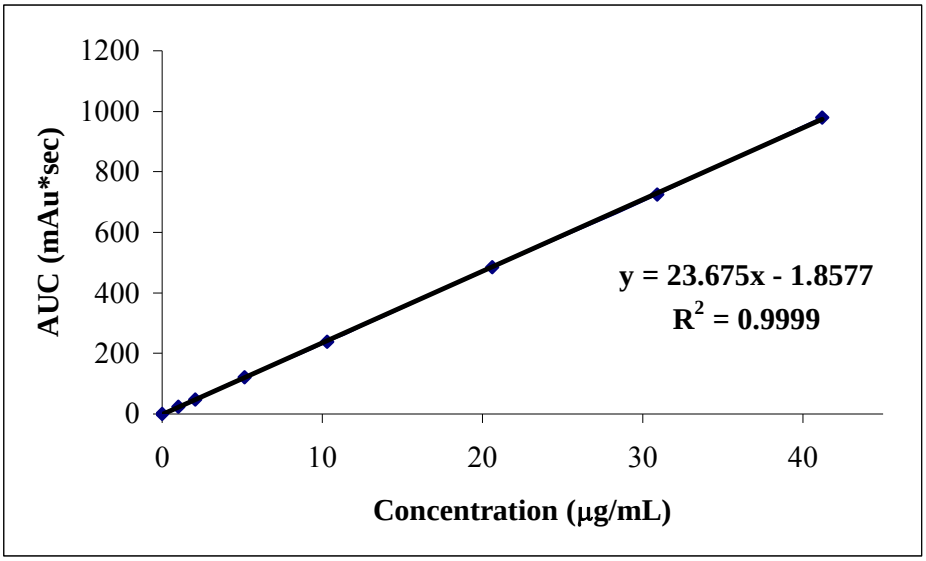


Figure 3.1: Standard curve for TA in ACN:mobile phase 1:1. TA was injected into HPLC in the range concentration of 1.03 µg/mL to 41.20 µg/mL, in 20µL injection volumes. Data is a representative of three independent calibration curves. r2 was calculated by Microsoft Excel™ software.

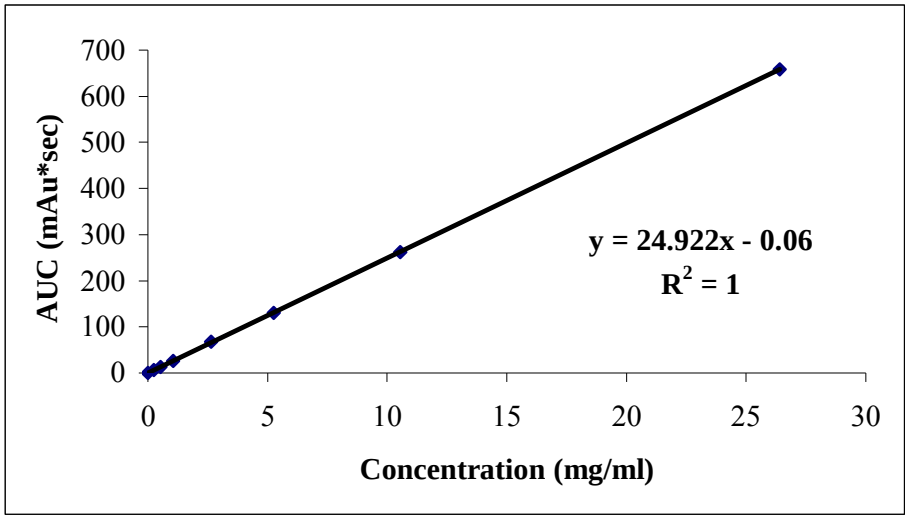


Figure 3.2: Standard curve for TA in PBS-NaN_3. TA was injected into HPLC in the range concentration of 0.264 µg/mL to 26.4 µg/mL, in 20µL injection volumes. Data is a representative of three independent calibration curves. r2 was calculated by Microsoft Excel™ software.

System precision for the HPLC assay is shown in Table 3.1. Six injections (20 µL volumes) were carried out at the concentration of 1.03 µg/mL and 30.90 µg/mL with ACN:mobile phase 1:1 as the medium. The relative standard deviation (RSD) of the injections was obtained below the nominal acceptable level of 2%. In the release medium, six injections of the concentration of 0.53 µg/mL and 5.27 µg/mL produced RSD of 0.53% and 0.08%, respectively, indicating that the injection of assay was precise for TA analysis.

Table 3.1: System precision of the HPLC method for TA determination

(a) 1.03 µg/mL of TA solution in ACN: mobile phase 1:1

Injection numbers	AUC (mAu*sec)
1	26.78
2	27.23
3	27.78
4	26.95
5	27.15
6	27.14
Average	27.17
RSD (%)	1.25

(b) 30.90 µg/mL of TA solution in ACN:mobile phase 1:1

Injection numbers	AUC (mAu*sec)
1	788.93
2	759.54
3	759.19
4	757.91
5	764.13
6	756.31
Average	764.33
RSD (%)	1.61

(c) 0.53 µg/mL of TA in PBS-NaN_3

Injection numbers	AUC (mAu*sec)
1	13.08
2	13.21
3	13.11
4	13.03
5	13.20
6	13.11
Average	13.12
RSD (%)	0.53

(d) 5.27 µg/mL of TA in PBS-NaN_3

Injection numbers	AUC (mAu*sec)
1	129.85
2	129.94
3	130.09
4	130.04
5	130.13
6	129.98
Average	130.00
RSD (%)	0.08

The accuracy of the HPLC assay was studied by examining the levels of recovery of TA in the presence of empty NPs made of three different polymers. The recovery of TA was investigated for determination of TA loading to assess the interference of the NP matrix in the assay. The results are presented in table 3.2.

Table 3.2: Recovery of TA in the presence of empty NPs in mobile phase

(a) PDLLA NPs as the empty NPs

Initial concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (%)
2.56	2.70	105.20
5.13	4.99	97.22
10.26	9.93	96.81
20.52	20.27	98.79
30.78	30.25	98.28
41.04	40.47	98.62
Mean ± SD		99.15 ± 2.79

Regression analysis	
Test	Results
Slope	0.9849
Intercept	-0.0069
Correlation coefficient	0.9999
95% CI slope	0.9738-0.9960
95% CI Y-intercept	-0.5187 to 0.5050

(b) PLGA NPs as the empty NPs

Initial concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (%)
2.56	2.67	103.97
5.13	5.07	98.85
10.26	10.09	98.38
20.52	20.15	98.20
30.78	30.49	99.06
41.04	40.91	99.68
Mean ± SD		99.69 ± 1.97

Regression analysis	
Test	Results
Slope	0.9941
Intercept	-0.00885
Correlation coefficient	0.9999
95% CI slope	0.9813-1.0070
95% CI Y-intercept	-0.6809 to 0.5036

(c) mPEG PLGA as the empty NPs

Initial concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (%)
2.56	2.69	104.89
5.13	5.14	100.23
10.26	9.97	97.20
20.52	20.21	98.49
30.78	30.55	99.24
41.04	40.78	99.38
Mean ± SD		99.91 ± 2.41

Regression analysis	
Test	Results
Slope	0.9921
Intercept	-0.0267
Correlation coefficient	0.9999
95% CI slope	0.9798 – 1.0045
95% CI Y-intercept	-0.5961 to 0.5427

In the presence of empty PDLLA NPs, the 95% confident interval (CI) of the slope was 0.9738-0.9960, not including 1 (Table 3.2.a). It indicates that the TA was not totally recovered by the assay method in the presence of the PDLLA matrix. However, the average of recovery was in the value of 99.69 ± 1.97%, with a slope and the correlation coefficient of 0.9849 and 0.9999, respectively. The 95% CI of Y-intercept goes through zero, therefore, this level of recovery was considered acceptable. Other tables, which show the recovery levels in the presence of empty PLGA NPs (Table 3.2 (b)) and empty 4.8% mPEG-PLGA NPs (Table 3.2 (c)), revealed 95% CI of slope including 1 and 95% CI of Y-intercepts going through zero. These data indicate that the assay is unbiased and produces a satisfactory recovery.

The recovery of TA in the release media was not carried out since there was no extraction method prior to TA determination by HPLC in the release study. Moreover, the release study was conducted using the dialysis bags and the samples were taken from the media outside the dialysis bag. The molecular weight cut-off (MWCO) of the dialysis bag was about 12,400 Da, therefore it would allow TA to freely pass through, while the NPs, whose size is much larger than MWCO, would be likely to be retained inside the bag.

In order to assess the sensitivity of the assay, the theoretical minimum LOD and minimum LOQ were determined both in the loading determination and in the release study. The theoretical LOD and LOQ for loading determination, which includes the extraction process of three different types of NPs, were approximately 0.107 μg/mL and 0.336 μg/mL, respectively (Table 3.3). In the release study, the HPLC assay for

TA determination was theoretically able to detect TA at levels above 0.053 µg/mL, and to provide accurate quantification of TA above 0.180 µg/mL. A sample of LOD and LOQ calculation is shown in appendix 1.

Table 3.3: LOD/LOQ of the HPLC assay for TA determination

	LOD (µg/mL)	LOQ (µg/mL)
Loading determination:		
- PDLLA NPs	0.090	0.299
- PLGA NPs	0.094	0.315
- mPEG PLGA NPs	0.118	0.394
Release study:	0.053	0.180

Selectivity in the HPLC assay development was assessed to determine that the peaks can be well-separated and the purity factor of the drug analysed is within the calculated threshold. The typical HPLC chromatograms of TA and degradation products are illustrated in Figure 3.3 from (a) to (f).

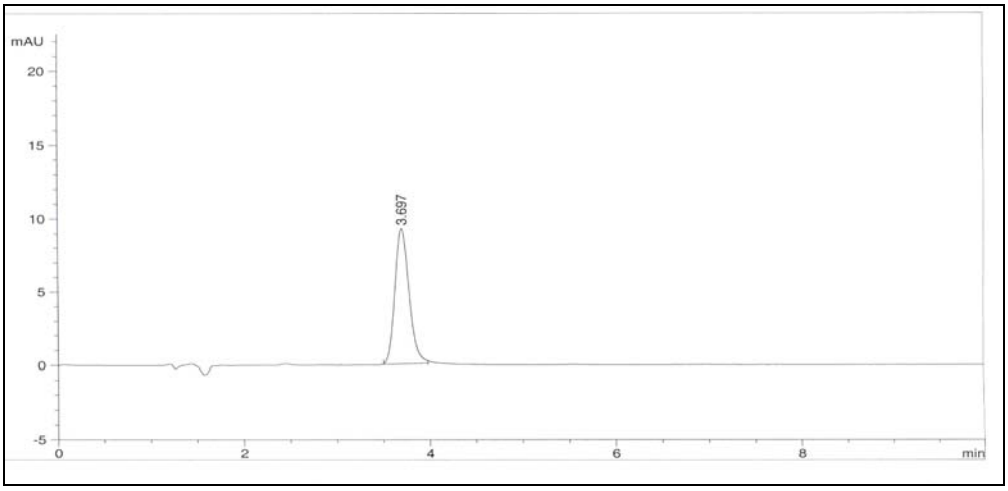


Figure 3.3: (a) Typical HPLC chromatogram of TA standard in ACN:mobile phase 1:1

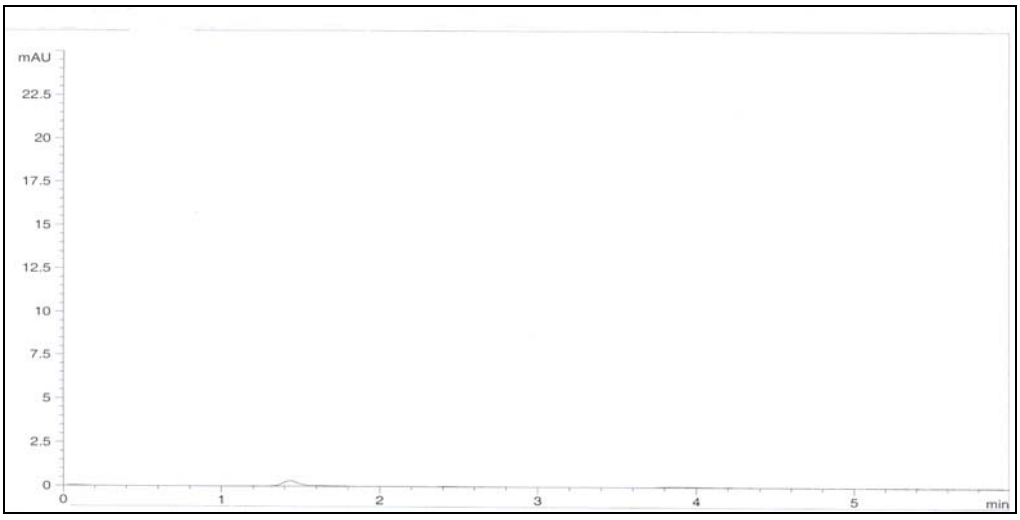


Figure 3.3: (b) Typical HPLC chromatogram of extraction of empty NPs in ACN: mobile phase 1:1 as blank

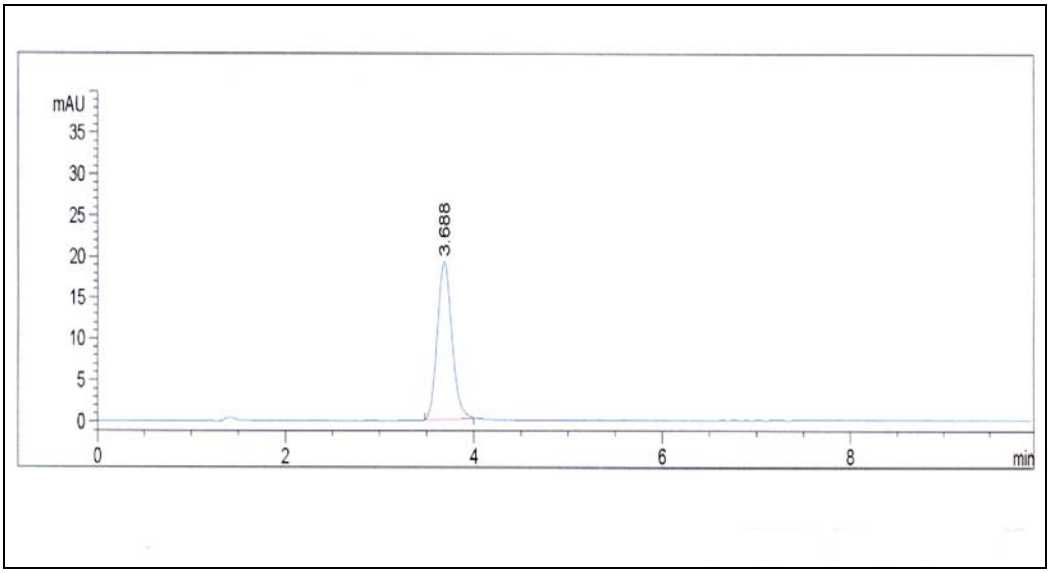


Figure 3.3: (c) Typical HPLC chromatogram of TA extraction from loaded NPs in ACN: mobile phase 1:1

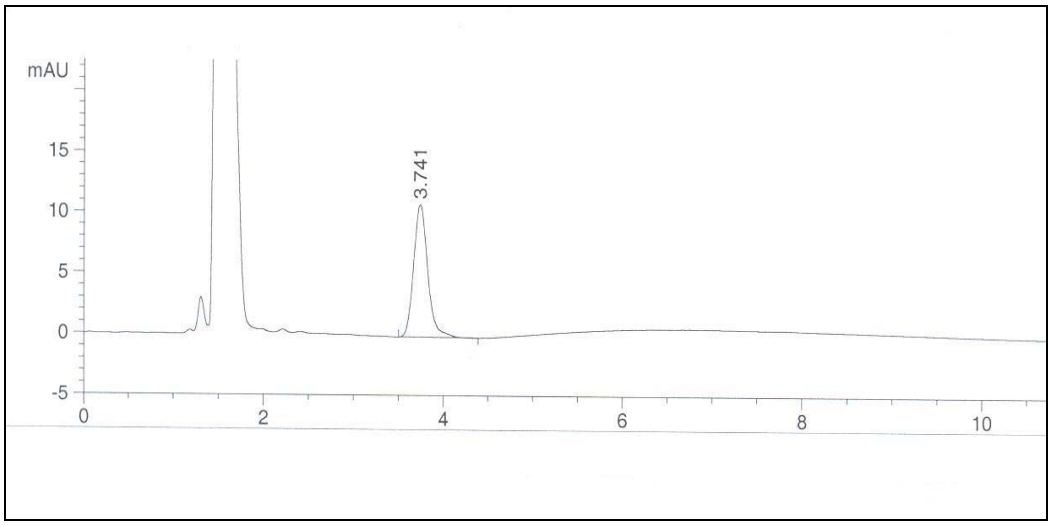


Figure 3.3: (d) Typical HPLC chromatogram of TA standard in PBS-NaN_3

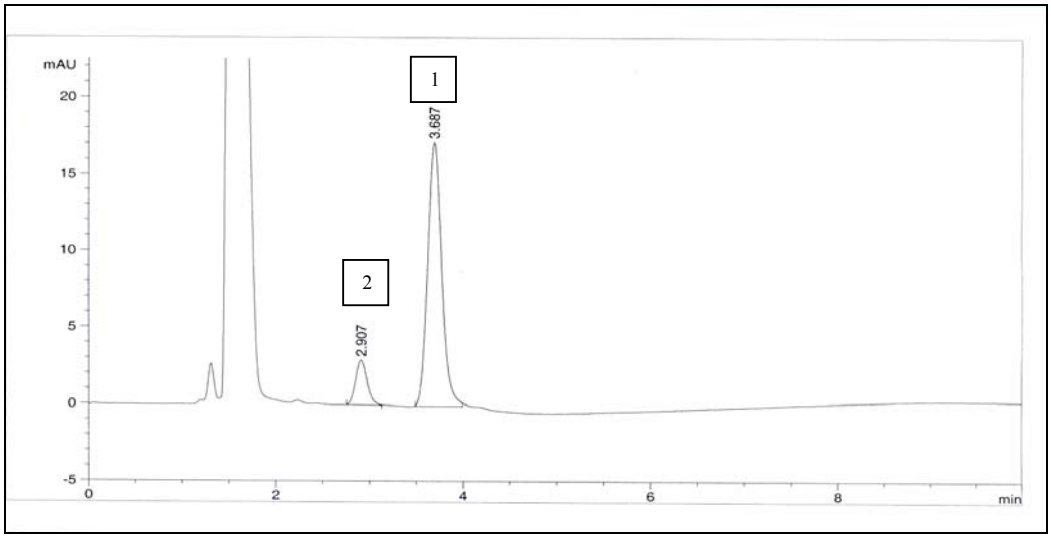


Figure 3.3: (e) Typical HPLC chromatogram of TA (peak 1) and degradation product (peak 2) in PBS-NaN_3 at 37°C over 24 hours

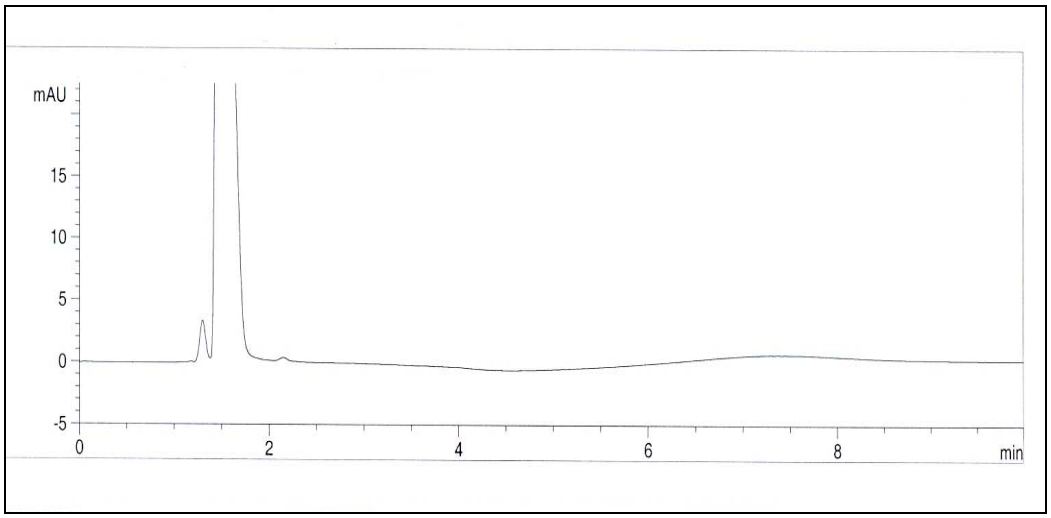


Figure 3.3: (f) Typical HPLC chromatogram of PBS-NaN_3 (release medium)

The retention time of TA and its degradation product (which is speculated to be 6β-hydroxyltriamcinolone[72]) were approximately 3.7 and 2.9 minutes respectively, showing that they were completely separated (Figure 33.(e)) In the release medium, the buffer substances and the sodium azide (NaN_3, a preservative) eluted faster than TA (at 1.6 minutes), suggesting that those compounds did not interfere with the TA analysis. Traces of PVA, in the loading determination were not detectable at a UV wavelength of 242 nm. In addition, the purity factor of the TA peak calculated by the Chemstation software package was within the calculated thresholds, indicating that the peak of TA was free from interference (see appendix 2).

3.2 Formulations of TA loaded NPs (TA-NPs)

The main objective of the current study was to develop NP formulations for TA which provide low toxicity, sustained release properties and prolonged stability. This study involved investigation of three different types of polymer, i.e poly- (D.L. lactide) (PDLLA), poly-(D,L,lactide-co-glycolide) (PLGA) and methoxy-poly ethylene-glycol-poly(D,L,lactide-co-glycolide) (mPEG PLGA) containing 4.8% polyethyleneglycol (PEG), for preparation of NPs. The solvent diffusion method was initially chosen to produce NPs, considering that it can produce particles sized around 200 nm using non toxic solvents and the production can be conducted in mild conditions [47-50, 57].

In the solvent diffusion method, acetonic solution containing polymer (for empty NPs) and polymer plus TA (for TA loaded NPs) was added dropwise into the PVA solution under mild agitation. The mixture of those solutions appeared to be opaque, since the NPs rapidly precipitated.

Table 3.4: Physical characteristics of empty NPs made by solvent diffusion method

Properties	PDLLA NPs	PLGA NPs	mPEG PLGA NPs
Particle size (nm)	284.93 ± 25.46	274.57 ± 5.09	212.77 ± 3.67
Polydispersity index	0.09 ± 0.02	0.07 ± 0.01	0.04 ± 0.01
Zeta Potential (mV)	-28.20 ± 3.24	-33.70 ± 0.90	-35.17 ± 3.47
Yield (%)	72.98 ± 8.71	84.38 ± 1.09	90.21 ± 1.91

All particles produced by the solvent diffusion exhibited small size, around 250 nm, with negatively charged surfaces (Table 3.4). About 80% yield was achieved by the solvent diffusion technique for empty NPs. The properties varied with nature of the polymers. Since the solvent diffusion technique involves a rapid diffusion of acetone into water, this rate of diffusion would affect the rate of formation and precipitation of NPs. From the results, it can be seen that the increased hydrophobicity of the polymer correlated with increased size.

Based on the properties obtained from empty NPs, incorporation of TA into NPs was carried out. Initially, it was found that the level of TA incorporated into PDLLA NPs appeared to be good (Table 3.5).

Table 3.5: Results of initial study of TA incorporation into PDLLA NPs by solvent diffusion

Batch No.	Particle size (nm)	Polydispersity index	Yield (%)	Loading (%)	Entrapment efficiency (%)
6	240.8	0.01	72.03	31.41	67.90
9	242.9	0.10	69.89	31.87	66.69
17	219.9	0.06	75.28	14.5	65.80

However, further investigation, conducted on the morphological characteristics of TA-PDLLA NPs, revealed that the NP suspension contained a mixture of TA-PDLLA NPs and TA crystals. Figure 3.4 shows a scanning electrone microscopy (SEM) image of TA crystals found in the initial TA-PDLLA NPs sample. We hypothesised that, although the polymer was able to form NPs immediately after being introduced to the PVA solution, TA was less entrapped in NPs during diffusion process, instead, most of TA precipitated in the PVA solution. This led to artificially high entrapment efficiency. To test this hypothesis, the next study was carried out.

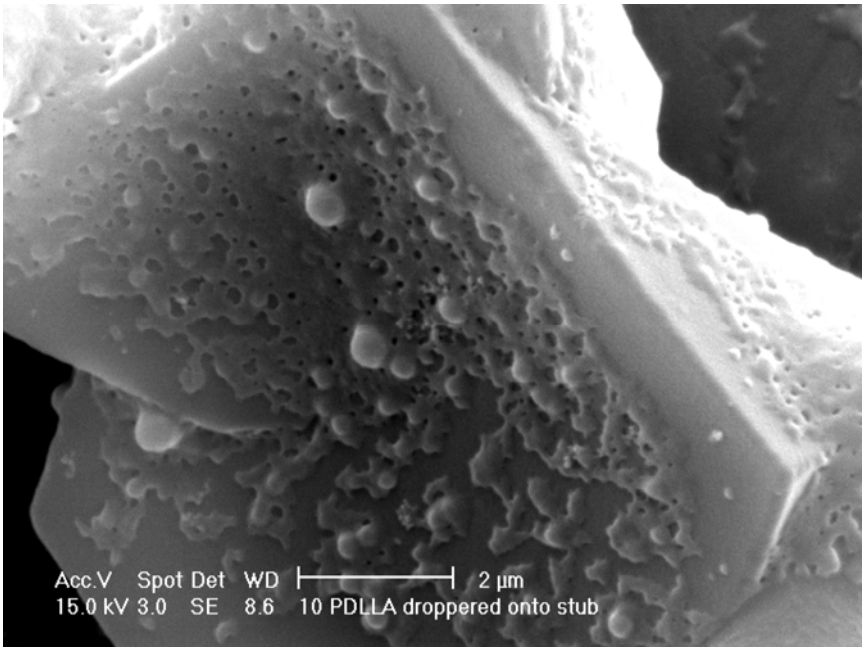


Figure 3.4: SEM of TA crystal in TA-PDLLA NPs.

Turbidity study

To assess the order of precipitation between polymer and TA during formation of NPs in the solvent diffusion method, the turbidity study was conducted on two types of polymer: PDLLA and mPEG-PLGA. The former has the most hydrophobic characteristics whereas the later is the most hydrophilic polymer used in this study. In this turbidity study, the experiment was designed similarly as for preparing the NPs, except that the polymer-acetonic solution and TA-acetonic solution were poured individually into PVA solution. The precipitation (turbidity of solution) was determined at a UV wavelength of 400 nm. A mixture of acetone and PVA solution was used as blank. Figure 3.5 shows that the polymers precipitated immediately after being introduced to the PVA solution, while TA took about five minutes to be precipitated. This suggests that only a small amount of TA might be incorporated into NPs due to the rapid precipitation of the polymers.

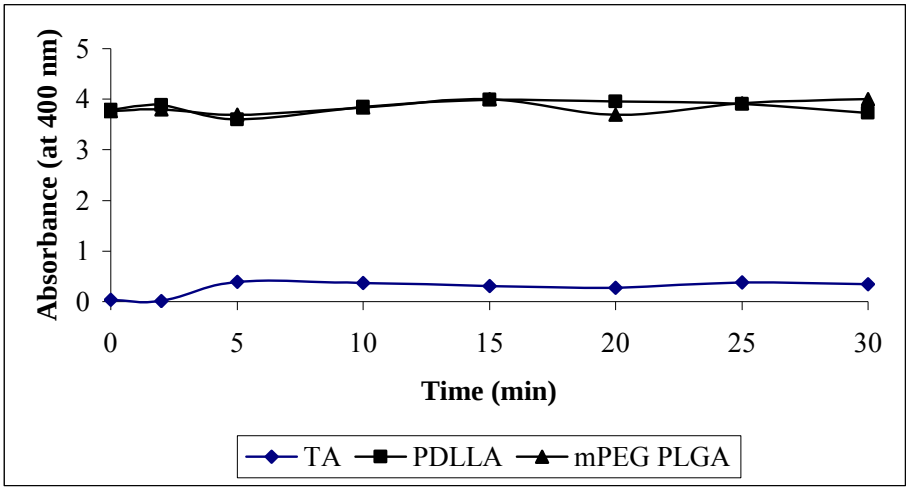


Figure 3.5: Turbidity profile of TA and polymers. The absorbance was evaluated at wavelength of 400 nm in certain time points during solvent diffusion process. The mixture of 0.6% PVA solution and acetone was used to correct the background

Modification of solvent diffusion method

The solvent diffusion method was modified to separate the TA crystals from TA-NPs produced by solvent diffusion process. In order to separate NPs from TA crystals, the NP suspension containing TA crystals was left on the bench for about 30 minutes. The settled TA crystals were then separated from NP suspension prior to centrifugation of NP suspension at 12,000 rpm for 20 minutes. The physical characteristics of the NPs prepared by this optimised method are shown in Table 3.6.

Table 3.6: Properties of TA-loaded- NPs made by the optimised solvent diffusion method

Properties	TA-PDLLA NPs	TA-PLGA NPs	TA-mPEG PLGA NPs
Particle size (nm)	256.7 ± 2.54	279.0 ± 19	233.63 ± 4.29
Polydispersity index	0.057 ± 0.005	0.040 ± 0.020	0.090 ± 0.001
Zeta Potential (mV)	-30.53 ± 0.95	-33.06 ± 2.31	-30.05 ± 1.20
Yield (%)	50.08 ± 4.27	60.04 ± 5.89	82.73 ± 1.90
Loading (%)	1.57 ± 0.73	2.47 ± 0.34	2.94 ± 0.25
Entrapment efficiency (%)	4.23 ± 1.79	7.91 ± 1.07	12.3 ± 0.87

In terms of the characterisation of NPs, the smallest particles were obtained in TA-mPEG PLGA NPs (around 230 nm), followed by TA-PLGA NPs and TA-PDLLA NPs (around 260 nm). The small size of TA-mPEG PLGA was due to the PEG content on mPEG PLGA. Beletsi et al [99] and Avgoustakis et al [54] suggest that PEG has the ability to mediate the association of polymer molecules during the formation of NPs. In terms of entrapment efficiency and loading value, we revealed that the entrapment efficiency and the load value of TA-NPs, especially TA-PDLLA NPs, were extremely low, around 1% and 5% respectively. However, the result of TA-PDLLA NPs particle size is in agreement with other study conducted by Liu et al [50]. They demonstrated that the particle size of PLA NPs of 231.5 ± 32.1 nm was obtained with the same polymer concentration (2%). Berton et al [100], in their study of incorporating oligonucleotide complex (a hydrophobic drug model) into PDLLA, revealed that the actual loading of only 2.91% w/w could be obtained for a theoretical loading of 10%. The failure to entrap the drug was possibly due to the rapid diffusion of the acetone to outer phase. As the mechanism of this method involves diffusion of solvent, [48, 58, 101], a rapid diffusion may cause the polymer to solidify rapidly. Due to the poor solubility of the hydrophobic polymers in aqueous stabiliser solution, polymer aggregation may occur at the same time with NP formation, resulting in a low level of yield of NPs. Leo et al [102] also suggested that a substantial decrease of yield with the increased of drug loading may be due to the

rapid diffusion of the drug to external aqueous phase during NP solidification. This suggestion was correlated to the study conducted by Govender et al [20]

Since the loading and entrapment efficiency of NPs prepared by the solvent diffusion method were undesirable, the second technique of NP preparation was studied. Emulsification-solvent evaporation, also known as solvent evaporation, is the most common method for preparing polymeric nano- and microparticles. In this method, droplet formation, droplet stabilisation and solidification of NPs after solvent evaporation play an important role in controlling the size of NPs [30]. The formation of NPs is achieved by evaporating the organic solvent which is immiscible with water. Although it involves organic solvent which is considered potentially toxic, the level of organic solvent can be reduced with a proper evaporation method.

DCM containing polymer (for empty NPs) and polymer plus TA (for TA-NPs) were emulsified in the PVA solution by sonication. The emulsion was then solidified under mild agitation prior to DCM evaporation by a rotary evaporator (Buchi, Germany). In the study of praziquantel loaded NPs, Mainardes and Evangelista [56] demonstrated that evaporation of the organic solvent using a rotary evaporator, with reduced pressure, offered smaller particles and a more efficient process with regards to drug entrapment, compared to the same process using a magnetic stirrer.

Table 3.7: Physical characteristics of empty NPs made by emulsification-solvent evaporation method

Properties	PDLLA NPs	PLGA NPs	mPEG PLGA NPs
Particle size (nm)	338.57 ± 17.75	292.38 ± 3.45	274.29 ± 11.88
Polydispersity index	0.11 ± 0.02	0.08 ± 0.01	0.060 ±0.005
Zeta Potential (mV)	-36.2 ± 1.60	-34.24 ± 1.27	-34.80 ± 0.82
Yield (%)	89.42 ± 3.00	91.03 ± 5.23	88.11 ± 3.95

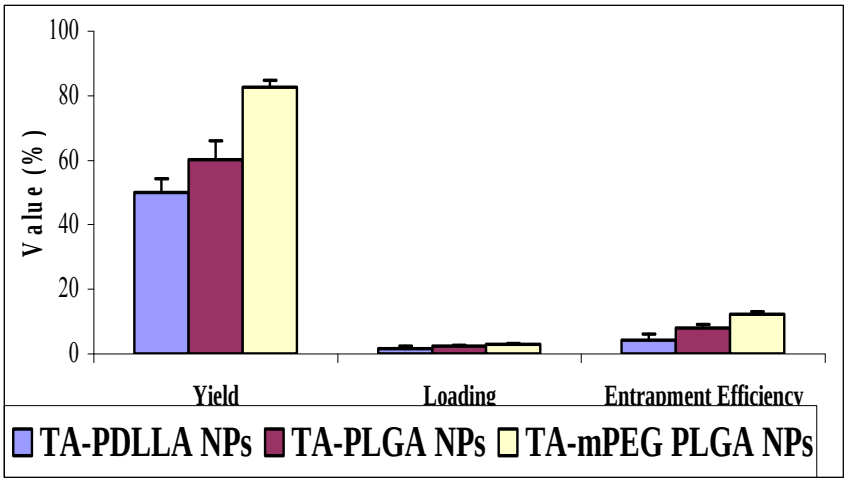
The particle size of three types of NPs, which varied with the type of polymer, was found to be larger than the formulations with the solvent diffusion method due to slow evaporation of DCM (Table 3.7). The surface charges were found to be highly negative, indicating that the NPs were in a stable suspension. Regarding the yield, this technique produced more NPs compared to the solvent diffusion method.

Incorporating of TA into NPs was also carried out by solvent evaporation method. As expected, the particle size of TA-NPs was larger than that of empty NPs.

Table 3.8: Properties of TA-loaded- NPs made by emulsification-solvent evaporation method

Properties	TA-PDLLA NPs	TA-PLGA NPs	TA-mPEG PLGA NPs
Particle size (nm)	360.35 ± 1.91	326.85 ± 0.35	291.35 ± 4.03
Polydispersity index	0.03 ± 0.02	0.030 ± 0.005	0.06 ± 0.03
Zeta Potential (mV)	-33.25 ± 8.56	-32.85 ± 6.43	-32.15 ± 2.76
Yield (%)	79.39 ± 1.86	92.30 ± 2.06	87.61 ± 4.05
Loading (%)	6.09 ± 0.85	12.00 ± 0.43	12.69 ± 0.85
Entrapment efficiency (%)	29.57 ± 4.55	67.26 ± 4.11	63.06 ± 1.69

(a)



(b)

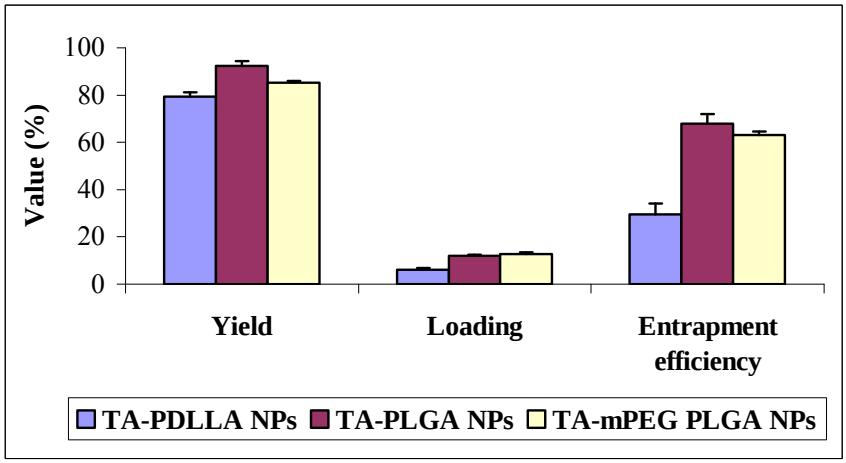


Figure 3.6: Comparison of yield, loading and entrapment efficiency values of three types of NPs. The NPs were made by the optimised solvent diffusion method (a) and solvent evaporation (b). The data is presented as mean ± SD (n=3). The error bars represent standard deviation of triplicate samples.

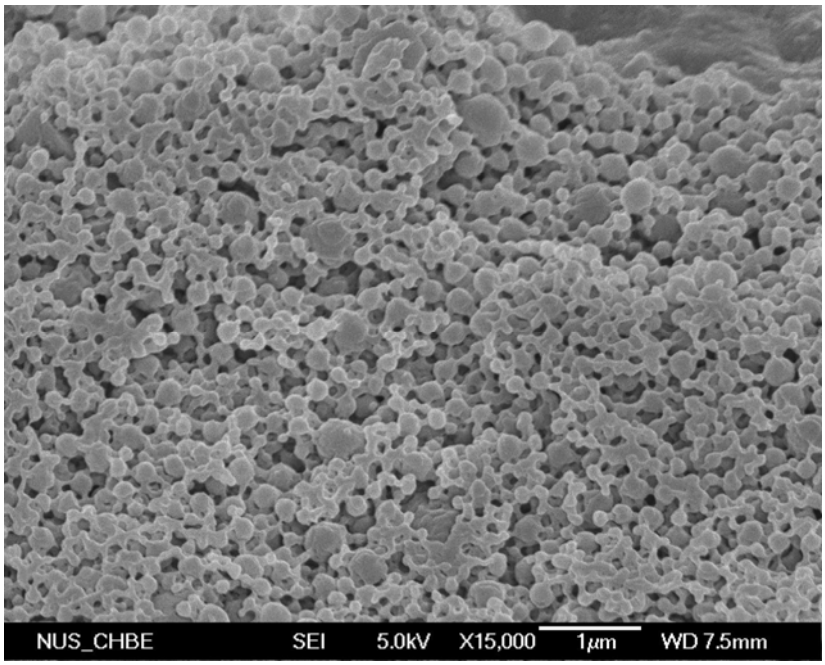
Although the particle size and zeta potential showed expected values, loading and entrapment efficiency of TA-NPs made by solvent diffusion method were out of expectation (Figure 3.6 (a)). The low drug entrapment efficiency and loading could be due to the rapid diffusion of acetone into water, which led to the rapid precipitation of polymers. Based on the obtained data, we can conclude that, when NPs are prepared by solvent diffusion method, a small quantity of the drug could be encapsulated leading to low entrapment efficiency. In other words, the solvent

diffusion method is only capable for producing NPs with a low entrapment efficiency.

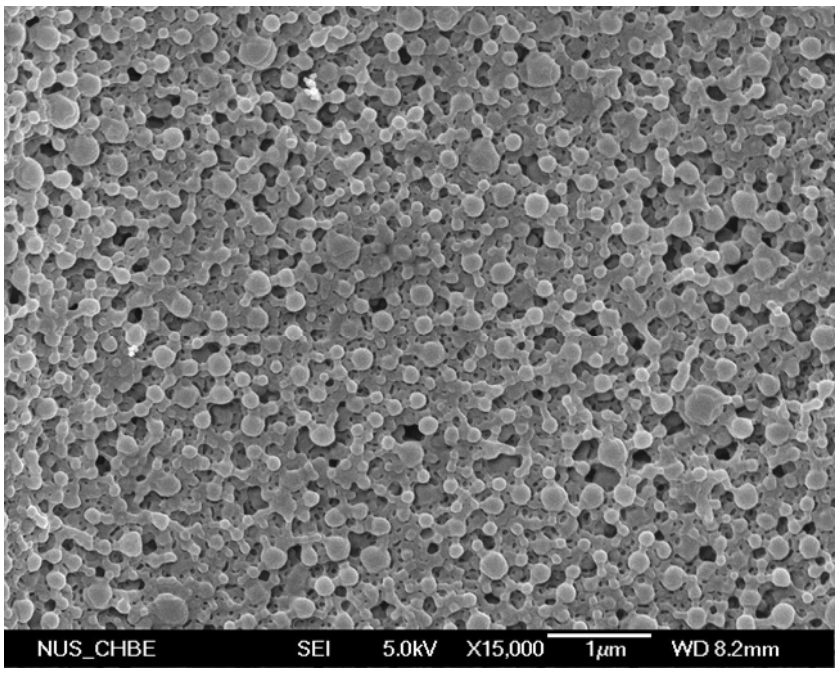
In solvent evaporation method, TA-NPs with substantial higher loading values could be achieved (Figure 3.6 (b)). The high drug loading and entrapment efficiency were achieved in both TA-mPEG PLGA NPs and TA-PLGA NPs. The yield of NPs was greatest with TA-PLGA NPs and TA-mPEG PLGA, but less with TA-PDLLA NPs. The incomplete recovery for TA-mPEG PLGA was due to the limitation of purification, in which the speed of centrifugation was not high enough to settle the mPEG PLGA NPs, resulting in loss of NPs during supernatant (PVA) removal. The purification of NPs will be further discussed as per section 3.5. Low levels of loading, entrapment efficiency as well as the yield of TA-PDLLA NPs were due to the formation of TA crystals, microparticles and polymer aggregation, which were separated from NPs prior to centrifugation.

The morphological characteristics of TA-NPs prepared by solvent evaporation were examined. Field emission scanning electron microscopy (FESEM) was used to capture images of TA-NPs.

(a)



(b)



(c)

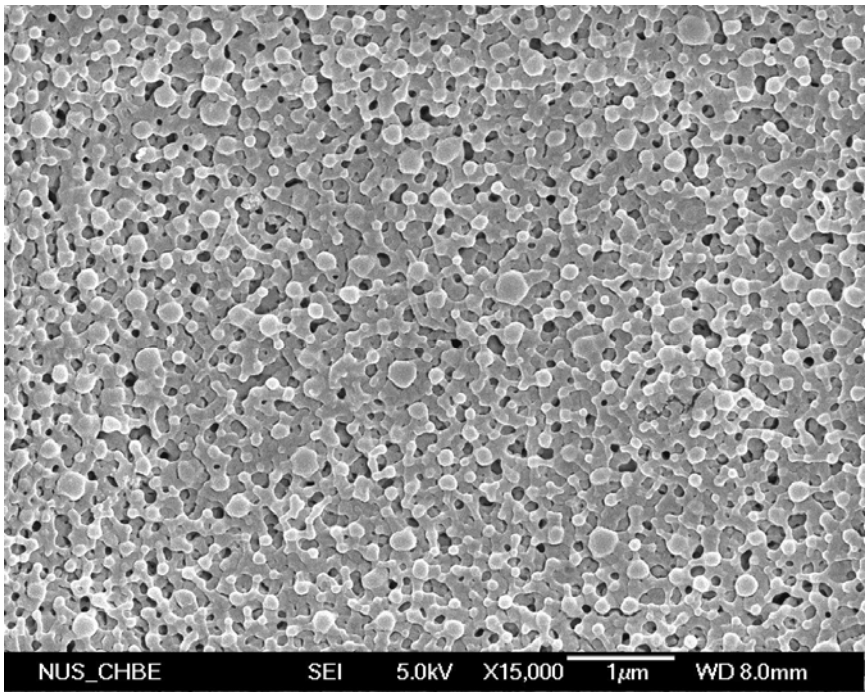
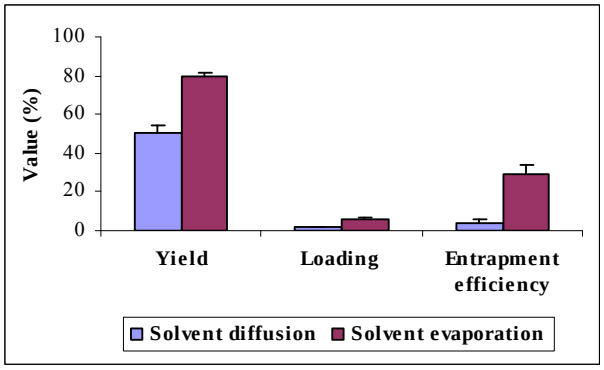


Figure 3.7: FESEM image of TA-NPs. TA-NPs were prepared by solvent evaporation method. (a) TA-PDLLA NPs, (b) TA-PLGA NPs, (c) TA-mPEG PLGA NPs. Images were taken at 15,000 magnification.

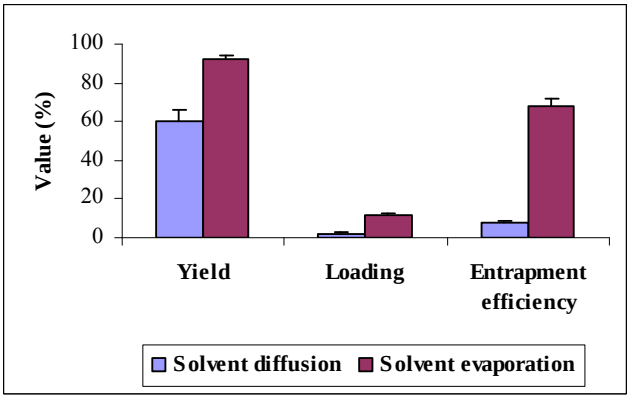
The morphological characteristics of TA-PDLLA NPs, TA-PLGA NPs and TA-mPEG PLGA made by emulsification solvent evaporation show that the NPs were spherical, with size less than 500 nm (Figure 3.7). No free TA crystals mixed with the NPs were detected. The connection that appeared in between NPs was maybe caused by the drying process or the presence of PVA residue on the surface of NPs. The size of the TA-PDLLA NPs appeared to be heterogeneous. In addition, TA-mPEG PLGA appeared to be slightly smaller than the other two types of NPs.

Those findings are correlated with particle size measurement by Zetasizer 3000HS (Malvern Instrument Pty. Ltd., Worcestershire, UK) which was based on dynamic light scattering. However, since the samples measured by Zetasizer™ was in a suspension, indicating that the NPs were fully hydrated, the particle size appeared to be larger than the FESEM samples, which have to be prepared in dry conditions. Due to time limitation, the size measurement on FESEM was only conducted on TA-mPEG PLGA NPs made by solvent evaporation method (appendix 6).

(a)



(b)



(c)

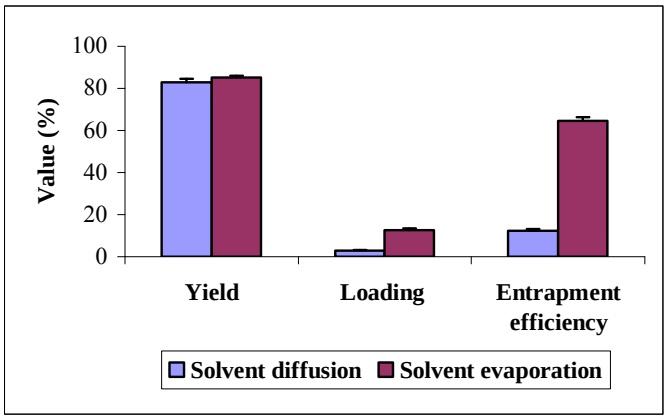


Figure 3.8: TA-NPs characterisation. The column chart illustrates the comparison of yield, loading and entrapment efficiency obtained in TA-NPs made by solvent diffusion and solvent evaporation methods (a) TA-PDLLA NPs, (b) TA-PLGA NPs, (c) TA-mPEG PLGA NPs. Data is presented as mean ± SD (n=3).

It is evident that in general, the solvent diffusion method can reduce the size to approximately 200 nm even less [47, 49, 50, 103]. In the solvent diffusion method, the particle size of TA-PDLLA NPs was 256.7 ± 2.5 nm, whereas in the solvent evaporation the TA-PDLLA NPs sized around 360.4 ± 1.9 nm. The large particles (>200 nm) formed was possibly due to the combination of hydrophobicity of polymer and a relatively high polymer concentration. Zeta potentials of approximately -30 mV were found in both method, indicating that the NPs were well-dispersed and the suspension was stable. Solvent diffusion method produced a low yield value with TA-PDLLA NPs (Figure 3.8). This was due to the formation of aggregates and microparticles during the preparation process, which were settled and removed prior to purification. The entrapment efficiency was found to be very low with the NP preparation by the solvent diffusion method. This may be due to the rapid diffusion of organic solvent resulting in very rapid solidification of the polymer (PDLLA). However, solvent evaporation can produce TA-PDLLA NPs with relatively higher entrapment efficiency.

The similar patterns, which show that solvent evaporation was superior than solvent diffusion, were also shown in TA-PLGA NPs and TA-mPEG PLGA NPs.

To determine the distribution of TA on the preparation of NPs, a mass balance study was conducted for all types of NPs (Table 3.9). The proportion of TA in the

supernatant was similar in all types of NPs, indicating that the quantity of TA in supernatant was controlled by the solubility of TA. Correlating with the result on entrapment efficiency, it was found that the greatest amount of TA precipitated was in TA-PDLLA NPs due to the poor entrapment of NPs.

Table 3.9: Distribution of TA inside NPs, in supernatant and precipitate in emulsification-solvent evaporation method

Type of NPs	Distribution of TA (%)		
	Inside NPs	In supernatant	Precipitate
TA-PDLLA NPs	37.01 ± 0.59	4.30 ± 0.45	54.62 ± 0.20
TA-PLGA NPs	78.52 ± 0.44	4.31 ± 0.21	12.91 ± 0.43
TA- mPEG-PLGA NPs	80.85 ± 0.28	4.09 ± 0.40	14.26 ± 0.61

3.2.1 NP purification by high speed centrifugation

Purification of the NPs was analysed to remove potentially toxic impurities, such as traces of organic solvents, aggregates, surfactants or stabilisers. Polyvinyl alcohol (PVA) should be removed from the system in the preparation of NPs, since it is considered to be undesirable. Although PVA is reported as an effective stabiliser, which can assist to produce NPs with reduced size and high entrapment efficiency [36, 104], it is also reported by other investigator that PVA is a suspected potential carcinogen [105] .

Various methods can be conducted to purify the NPs such as tangential flow filtration, diafiltration centrifugal devices (DCD) and high speed centrifugation [62]. High speed centrifugation was selected as the method of purification for this study since it offered a simple and effective way to remove PVA. This method was carried out by centrifuging the NP suspensions at 12,000 rpm for 20 minutes at room temperature followed by decanting the PVA-containing-supernatant.

We adopted the method reported by Dalwadi et al [62] to determine the level of PVA in the system before and after purification, in order to analyse the efficiency of removal.

The method is based on the complexation of two adjacent hydroxyl groups of PVA and an iodine molecule, in the presence of boric acid. The green coloured complex produces an intense absorbance band in the visible spectrum region at a wavelength of 690 nm. Figure 3.9 shows a representative of a calibration curve which was used to detect and quantify the amount of PVA remaining in the supernatant. A regression coefficient (r^2) of 0.9964 was obtained, indicating that the absorbance was found to be linear with the concentrations of PVA, ranging from 19 to 67 µg/mL. A blank of the reagents excluding the PVA was used as zero concentration of PVA.

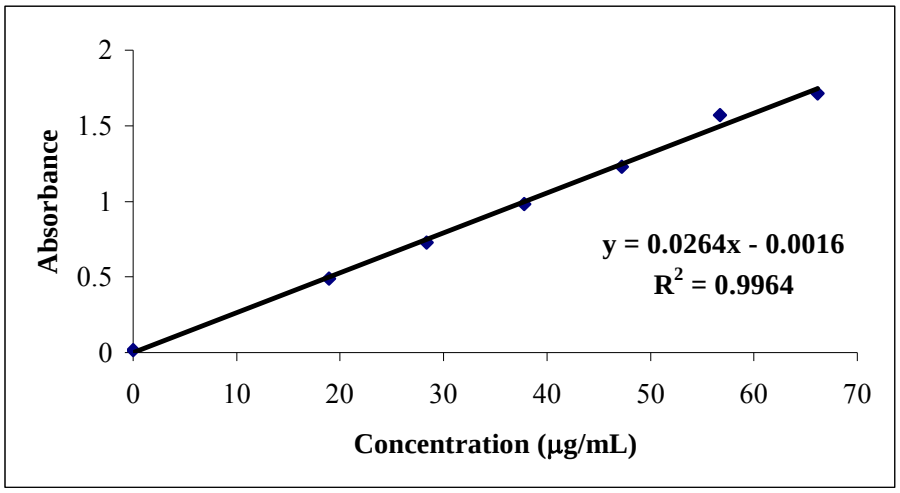


Figure 3.9: A representative of calibration curve of PVA determination. The curve was plotted on absorbance at 690 nm against PVA concentration in the range concentration of 19 to 67 µg/mL.

Although high speed centrifugation offers satisfactory results in removing the PVA (Table 3.10), PVA still could not be 100% removed from the system. This finding is corresponding with the study reported by Murakami et al [48, 106] which revealed that small amount of PVA appeared to be attached on the surface of NPs as a result of hydrophobic bonding.

Table 3.10 : PVA removal on purification of NPs by high speed centrifugation

Type of NPs	PVA removed (%)	
	Solvent diffusion method	Solvent evaporation method
TA-PDLLA NPs	94.28 ± 0.97	95.63 ± 0.37
TA-PLGA NPs	96.62 ± 0.20	96.61 ± 1.83
TA-mPEG PLGA NPs	96.66 ± 0.62	95.51 ± 2.03

Effect of purification on particle size

It is imperative to ensure that the method of purification does not affect the physical characteristics of NPs. To investigate the effect of purification on particle size, the particle size of the NPs before and after purification was analysed (Table 3.11).

Table 3.11: Particle size of the NPs before and after purification

Type of NPs	Before purification		After purification	
	Particle size (nm)	Polydispersity index	Particle size (nm)	Polydispersity index
PDLLA NPs	307.87 ± 4.28	0.10 ± 0.02	324.77 ± 1.44	0.12 ± 0.02
PLGA NPs	333.63 ± 1.33	0.07 ± 0.03	355.97 ± 2.12	0.06 ± 0.03
mPEG-PLGA NPs	230.80 ± 7.92	0.09 ± 0.02	233.63 ± 4.29	0.03 ± 0.02

A slight increase in particle size was found after purification, to the extent that there was no difference on particle size observed on mPEG-PLGA NPs. Polydispersity index of three different types of NPs appeared to be similar before and after purification. Therefore, the effect of high speed centrifugation is negligible, suggesting that high speed centrifugation is an effective purification methods for NPs.

3.2.2 Release characteristics of TA-NPs

To investigate the characteristics of TA-NPs in providing sustained release, an *in vitro* release study of the NPs was conducted by dialysis bag diffusion method designed as per section 2.3.6. Sodium azide (NaN_3, 0.05%) was added as a preservative in the release medium.

TA solubility in the release media was investigated prior to the release study, to determine the maximum concentration for maintenance sink conditions in the system. The design of solubility study can be seen on section 2.3.6. In this solubility study it was found that the solubility of TA was 6.58 ± 1.20 μg/mL over 8 hours, and 27.40 ± 0.40 μg/mL over 24 hours (appendix 7). In the release study, since the medium (PBS-NaN_3) was replaced with the fresh one every hour over 8 hours and every 24 hours over 96 hours (in sampling time), it can be sure that the sink condition was achieved and TA solubility issues did not contribute to the release of TA from NPs .

To develop a control, two TA suspensions in water containing a known amount of TA in different particle size, were used to examine the release profiles of free TA. Due to the limitations of instrumentation, the controls were only designed in micro-size. The first control was made by a similar solvent evaporation procedure so as to make the NPs, excluding the polymer and PVA. As can be predicted, this method produced a TA suspension with the microparticles sized around 5.34 ± 0.25 μm. In an attempt to reduce the size, the second control was established by sonicating the TA suspension for 5 minutes at room temperature. The size was found to be smaller than the first control, i.e. 2.86 ± 0.62 μm.

Although it was not possible to design a control group which has the same particle size as the NP samples without an excipient or a stabiliser, it can be seen that the release of TA was more rapid with the reduction of size (Figure 3.10). It was speculated that if we can design the control group with nano-sized particles, the release of TA would be much faster.

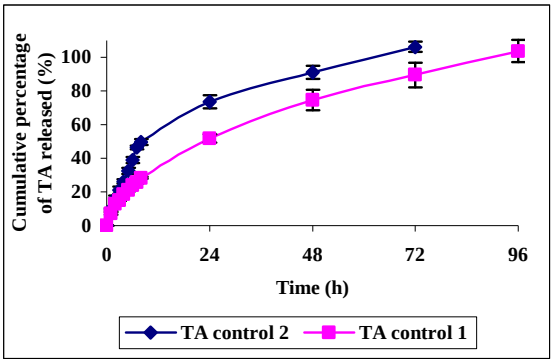
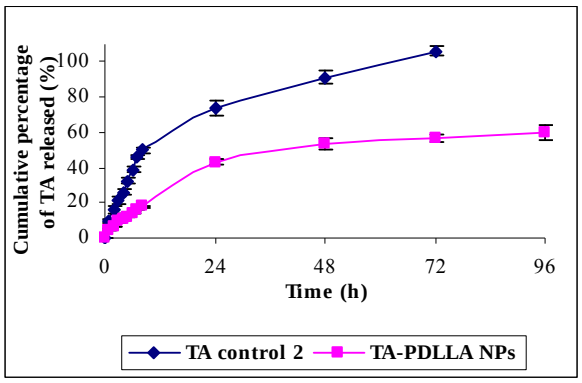


Figure 3.10: Release profile of TA control groups over 96 hours. Release study was conducted at 37°C using a dialysis-diffusion bag method in the PBS-NaN3 as the release media. TA control 1 was prepared by the same method of solvent evaporation for NPs (5.34 ± 0.25 μm). TA control 2 was prepared by sonicating TA in water for 5 minutes in the absence of stabiliser (2.86 ± 0.62 μm). Both control groups containing the same concentration of TA as in the NPs. Data is presented as mean ± SD (n=3).

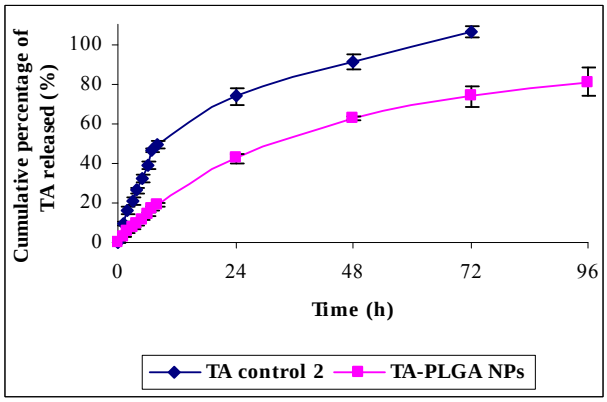
Nevertheless, because the system was not supported by any surfactants and, the particles of TA in the control groups aggregated over time, resulting in the smaller surface area, and hence the rate of release was reduced.

Release characteristics of TA-NPs

(a)



(b)



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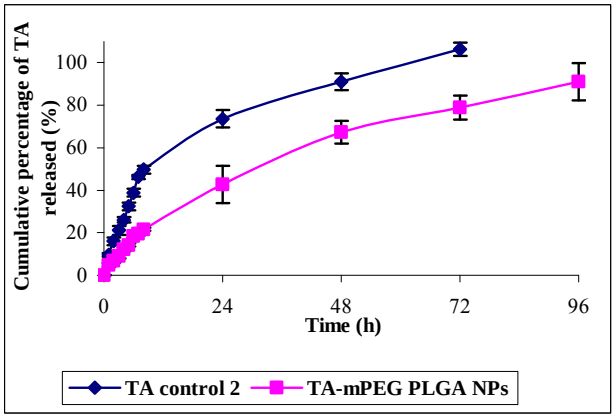


Figure 3.11: Release profile of TA-NPs over 96 hours. Release study was conducted at 37°C using a dialysis-diffusion bag method in the PBS-NaN3 as the release media, (a) TA-PDLLA NPs (b) TA-PLGA NPs (c) TA-mPEG PLGA. TA control 2 was prepared by sonicating TA in water for 5 minutes in the absence of stabiliser (2.86 ± 0.62 µm). Both control and NPs contain the same concentration of TA. Data is presented as mean ± SD (n=3).

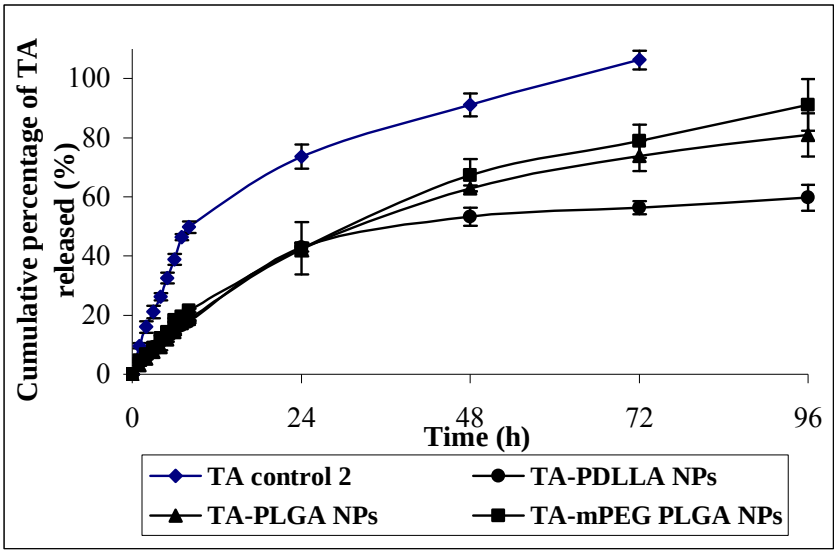


Figure 3.12: Overall release profiles of TA from TA-NPs over 96 hours. Release study was conducted at 37°C using dialysis-diffusion bag method in the PBS-NaN3 as the release media. TA control 2 was prepared by sonicating TA in water for 5 minutes in the absence of stabiliser (2.86 ± 0.62 μm). Both control and NPs contain the same concentration of TA. Data is presented as mean ± SD (n=3).

The release profiles of TA from TA-NPs were found to be biphasic (Figure 3.11 and 3.12). An fast constant release was observed over initial 8 hours (up to 20%) at a rate slower than TA control, indicating that TA was entrapped inside NPs and was dissolved and diffused out of NP matrix. This was followed by a sustained release up to 96 hours due to lower concentration gradient. Less than 60% of TA released over 96 hours from TA-PDLLA NPs, while TA-PLGA NPs released around 80% TA over 96 hours. TA was released slightly faster from TA-mPEG PLGA NPs than TA-PLGA NPs. Around 90% of TA was released from TA-mPEG PLGA NPs. Hans and Lowman [12] reported that large particles would exhibit longer sustained release than small ones. They also illustrated that in terms of loading, the higher the loading may cause an increase in release rate. In our study, the release characteristics were mainly due to the nature of the polymers. In comparison with the control, TA-NPs exhibited slower release with decreasing TA released over time. This indicates that all three types of NPs exhibited sustained release characteristics.

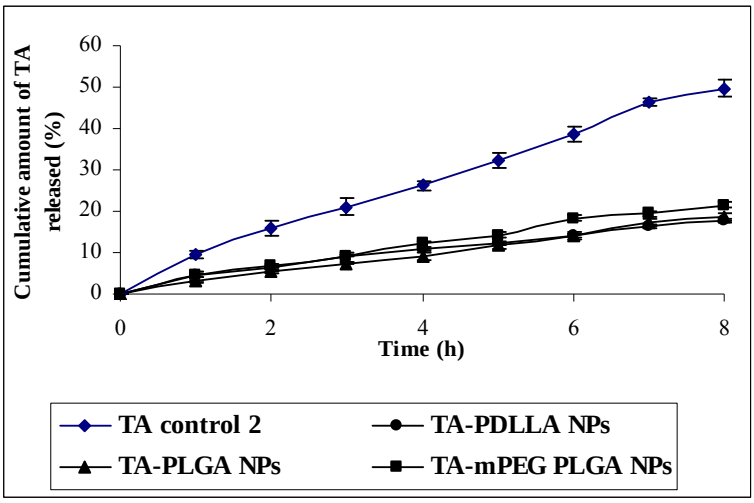


Figure 3.13: Release profiles of TA from TA-NPs over 8 hours. Release study was conducted at 37°C using dialysis-diffusion bag method in the PBS-NaN3 as the release media. TA control 2 was prepared by sonicating TA in water for 5 minutes in the absence of stabiliser (2.86 ± 0.62 μm). Both control groups containing the same concentration of TA as in the NPs. Data is presented as mean ± SD (n=3).

Release profiles of TA from TA NPs at initial 8 hours are presented in Figure 3.13. All three types of polymer show fast release at a constant rate up to 20% due to the dissolution and diffusion of TA which was entrapped inside NPs, to the media. Although all types of NPs show an initial fast release, the cumulative TA released was about half that of the control.

Table 3.12: Distribution of TA after release process over 96 hours

Distribution	TA proportion (%)			
	TA-PDLLA NPs	TA-PLGA NPs	TA-mPEG PLGA NPs	TA (control)
TA released over 96 h (in the release medium)	59.74	80.93	91.13	100*
Unreleased TA (in the NPs)	37.85	17.46	9.17	-
Total	97.59	98.39	100.3	

*released in 72 hours

Table 3.12 shows the distribution of TA after release over 96 hours. The unreleased TA was determined by the same method used for drug loading determination (section 2.3.4.4) which was extracting the TA by dissolving the NPs into ACN. From that

distribution, it can be concluded that only TA-PDLLA NPs exhibited true sustained release, probably due to the more rigid of NPs.

In summary, the difference of physical characteristics between TA-NPs in both methods was due to the nature of polymer and the solvent system used in the methods. The solubility of the drug and polymer in organic phase plays an important role especially in determining the entrapment efficiency. In the solvent diffusion, TA appeared to be very soluble in acetone. This led most of TA to diffuse out together with the organic phase to the aqueous phase. Although both TA and polymer are hydrophobic, it appeared that the interaction between TA and polymers was overweighed by the TA solubility in acetone. In the preparation of TA-NPs using the solvent evaporation the DCM was used as the organic solvent. In general we revealed that the particle size of TA-NPs was larger than those prepared by the solvent diffusion method. This was due to the slow evaporation of DCM. We also revealed that TA-PDLLA NPs were the largest particle with the lowest entrapment efficiency, while TA-mPEG PLGA NPs were the smallest particle with the highest entrapment efficiency. The release characteristics show that TA-mPEG PLGA NPs exhibited the fastest release of TA while the slowest release was shown in TA-PDLLA NPs. These results led to the speculation that although the amount of TA associated with PDLLA was low, association of TA with PDLLA appeared to be the strongest. PDLLA was likely to entrap TA thoroughly rather than other NPs. In the TA-mPEG PLGA NPs, the PEG presence on mPEG-PLGA enhanced the hydrophilicity of polymer, resulting in the relatively higher water uptake into NPs. This was probably the cause of the fastest release of TA.

3.2.3 TA stability study

One of the NP advantageous features is that they can protect the drug from hazardous environment and from potentially premature degradation [18]. To determine whether the NP formulations could enhance the stability of TA in the aqueous environment, an investigation of the stability of TA in the aqueous medium was carried out. In this study, the potency of TA in aqueous solutions was determined over 72 hours after storage at three different experimental temperatures: 4°C, 25°C, and 37°C. The potency of TA was calculated as a percentage of the TA peak area

over the total area of peaks (peaks of TA and degradation products). To exclude the possibility that limited drug solubility could reduce drug potency, both potency and degradation product were determined in each sample. In addition, the stability of TA inside the NPs was also investigated. The data of stability study are shown in appendix 3 and 4.

Stability of TA in the extraction medium of ACN: mobile phase 1:1

TA stability in the extraction medium was analysed using the HPLC assay of TA described in section 2.3.1. The results show that TA degraded slightly faster at higher temperatures (Figure 3.14). Although Gupta [74] reported that TA is likely to be unstable at pH above 5.5 in an aqueous medium, a slow process of the degradation was also observed in the aqueous medium with pH approximately 7.

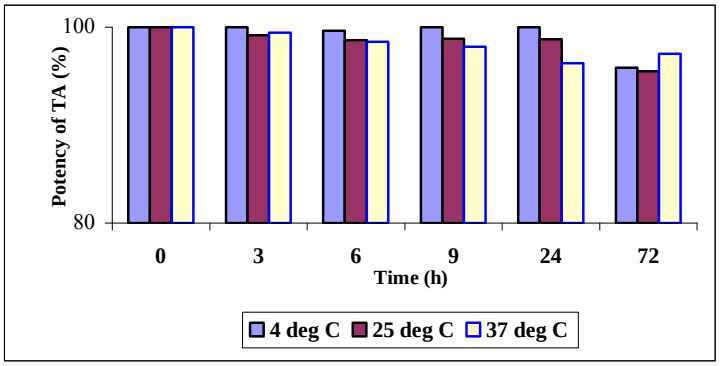


Figure 3.14: Potency of TA over 72 hours. Samples with a known concentration of TA in water were kept under experimental temperatures of 4°C, 25°C, and 37°C for 96 hours**.**

Stability of TA in the release medium

The stability of TA in the release medium was assessed at 4°C, 25°C, and 37°C using HPLC assay as per section 2.3.1. To investigate whether the stability of TA is affected by microbial activity, the study was conducted in the release media, with and without a preservative. Figure 3.15 presented the potency of TA in the phosphate buffer solution (PBS) pH 7.4 with and without a preservative. It was found that the change of potency of TA appeared to be insignificant affected by the inclusion of preservative in the release media over 72 hours. It may suggest that microbial activity does not influence the degradation process of TA.

In the release medium, TA was degraded the fastest at 37°C, as predicted. After 72 hours storage at 37°C, the degradation was increased dramatically up to 43%. A substantial decomposition of TA was found at room temperature, with TA potency reduced to 85% over 72 hours, while at 4°C the TA showed less than 10% degradation (6.4%).

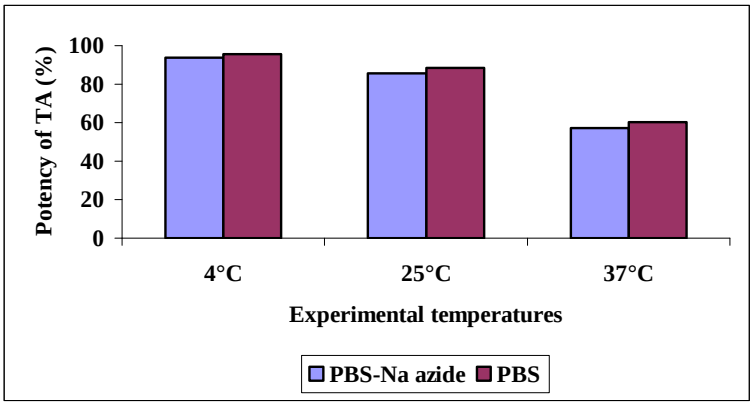


Figure 3.15: Potency of TA at 72 hours. Samples with a known concentration of TA in PBS and PBS–Na azide (PBS-NaN3) were kept under experimental temperatures of 4°C, 25°C, and 37°C for 96 hours.

TA stability inside NPs

To determine whether the NP formulations are able to maintain the stability of TA, the TA stability inside NPs was investigated. The study was conducted after completing a 96 hour *in vitro* release study. The TA was extracted from the NPs and analysed for its concentration as per section 2.3.4.4. Since TA in the control groups was 100% released in 72 hours, the potency of TA in the control groups was calculated with regards to 72 hours release. It was shown by the chromatogram (Figure 3.16) that after 96 hour release study at 37°C, TA remained in nature form inside NPs and no degradation product was detected. However, the free TA in the control groups was degraded up to approximately 12% over 72 hours. The data obtained suggests that the stability of TA was maintained satisfactorily by the polymer matrix of the NPs.

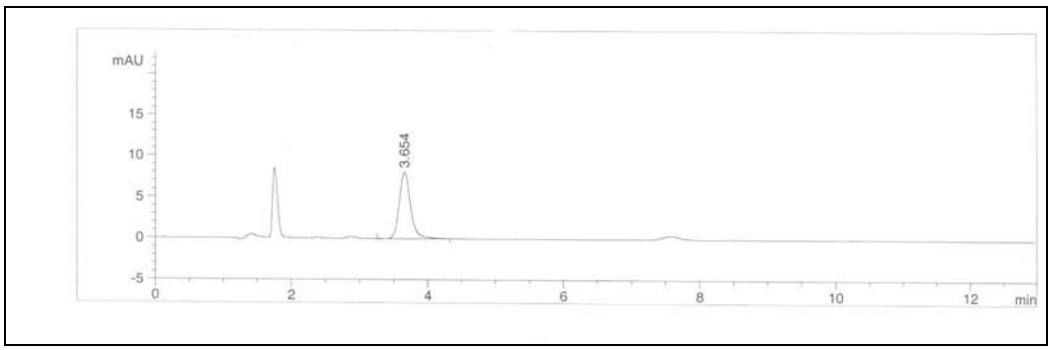


Figure 3.16: A representative of HPLC chromatogram of TA extraction from NPs. The extraction was carried out after 96 hour release study in PBS-NaN_3 at 37°C.

3.3 *In vitro* toxicity study of TA-loaded NPs

The issues of toxicity of NPs have been raised up recently, in accordance with the potential clinical application of NPs. To investigate the effect of either TA or TA-NPs, an *in vitro* toxicity study was carried out upon two different cell culture models: BALB/c 3T3 (fibroblasts) and ARPE 19 cells (human retinal pigment epithelial). The MTT assay was selected to determine the cell viability since it is a simple yet accurate assay [65], and is widely used in studies investigating polymers [44] and NP toxicity[107-109]. The MTT assay was well-established in this laboratory. Previous research in this laboratory has found that the MTT assay is the most efficient at demonstrating toxic effects on cell viability if the final absorbance of the untreated cultures is in the range of 0.7-1.2.

3.3.1 Assay optimisation

In order to effectively show adequate responses to toxic compounds, it was necessary at the first stage, to optimise the seeding density (number of cells per well) in the 96 well plates assay. The process to optimise the cell number per well is described as per section 2.3.8 using the MTT assay. BALB/c 3T3 cells were seeding with seeding density in the range of 5,000 – 20,000 cells/well at three different plates reflecting three different time points: 0, 24 and 48 hours (Figure 3.17). The optimum seeding density for BALB/c 3T3 was determined at 15,000 cells/well based on the linearity

of the response (absorbance) against time and final absorbance was in the range 0.7-1.2.

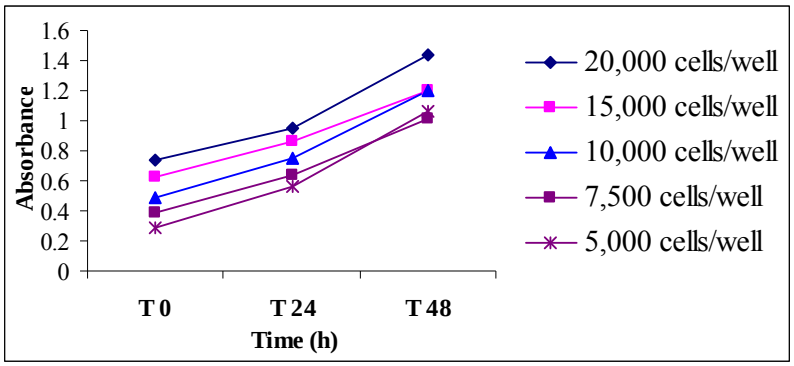


Figure 3.17: Seeding density optimisation of BALB/c 3T3. The cells were inoculated in D10 media in a range of seeding density and incubated at 37°C. The cell viability was assayed by MTT assay at each time point. Data shown is a representative of three independent studies.

Seeding density optimisation for ARPE 19 was also conducted as per section 2.3.8, with the same aim as that of BALB/c 3T3 (Figure 3.18). A range of seeding density was initially designed from 5,000 to 20,000 cells/well. In contrast to BALB/c 3T3 expression, ARPE 19 demonstrated low intensity of absorbance at the same incubation time. This may indicate that mitochondrial activity of ARPE 19 cells was not as active as BALB/c 3T3

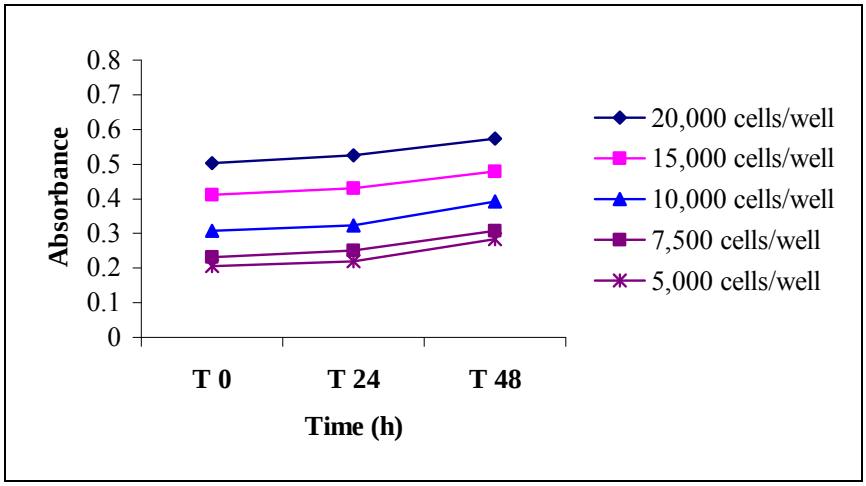


Figure 3.18: Seeding density optimisation of ARPE 19 (1). The cells were inoculated in DF12 media in seeding densities ranging from 5,000 to 20,000 cells/well and incubated at 37°C. The cell viability was assayed by MTT assay at each time point. Data shown is a representative of three independent studies.

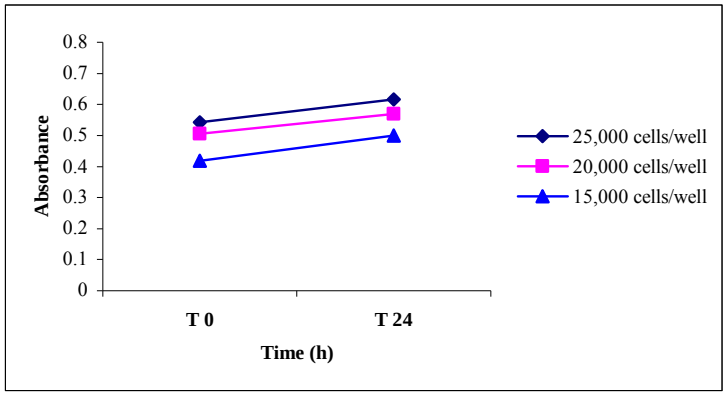


Figure 3.19: Seeding density optimisation of ARPE 19 (2). The cells were inoculated in DF12 media in seeding densities ranging from 15,000 to 25,000 cells/well and incubated at 37°C. The cell viability was assayed by MTT assay at each time point. Data shown is a representative of three independent studies.

In order to investigate if higher seeding densities were required for ARPE 19, a second experiment was carried out with 15,000 to 25,000 cells/well over 24 hours. As there was little improvement in the assay parameter at higher cell numbers, 20,000 cells/well was selected for ARPE 19 toxicity studies (Figure 3.19).

Interference of the test compounds/preparations on the MTT assay should be minimised in order to establish a valid assay. To investigate the possibility of such interference, an MTT assay was carried out with and without cells. In the absence of cells, the absorbance of residual samples was much lower than those of the presence of cells (Table 3.13). This indicates that the interference due to preparations or drug was minimal.

Table 3.13: Absorbance of samples with and without cells at 585 nm

Samples	Absorbance	
	With cells	Without cells
TA suspension (1 mg/mL)	0.673 ± 0.067	0.013 ± 0.002
TA-PLGA NPs with equivalent concentration of TA	0.754 ± 0.094	0.017 ± 0.005
Empty PLGA NPs with equivalent amount of NPs as of TA-PLGA NPs	0.865 ± 0.030	0.015 ± 0.04

3.3.2 BALB/c 3T3 cell viability assessment

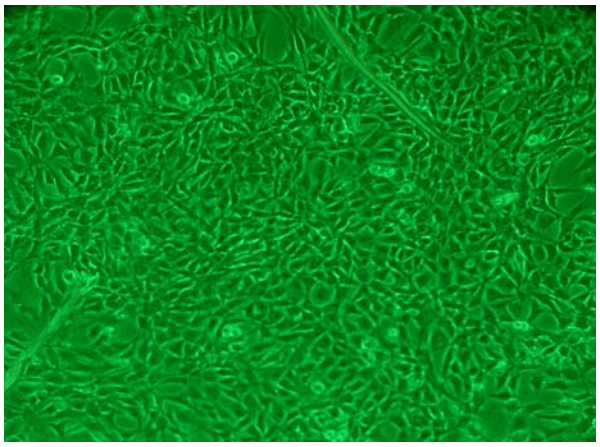
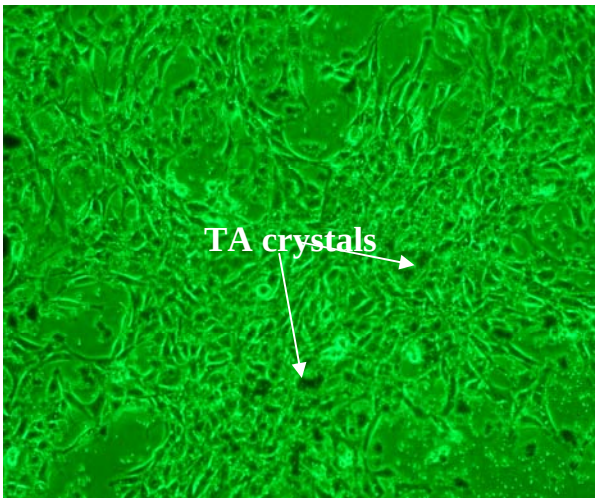


Figure 3.20: Image of BALB/c 3T3 cells. Image was taken with an aid of a phase contrast microscope at 33x magnification. The cells were inoculated in D10 media (80% confluence).

BALB/c 3T3 is the most common cell culture models used in toxicity study. Its feature of non tumorigenicity makes this cell model appropriate to study the toxicity of drugs which are not intended as anticancer agents. In order to assess the toxicity of TA and TA-NPs upon BALB/c 3T3, the study was carried out by exposing the cells to either TA or TA NPs over 24 hours at 37°C. The assessment was designed as per section 2.3.8.7. The range of TA concentration was designed from 0.1 µg/mL to 1 mg/mL based upon previous TA toxicity study conducted on ARPE 19 cells [98]. The TA-NPs were prepared to give an equivalent concentration of TA. Empty NPs were prepared to give an equal amount of NPs to TA-NPs. In addition to MTT assay, treated and untreated cell cultures were also observed by phase contrast microscopy. To investigate the condition of the cells after exposure, phase contrast microscopy was used. An image of cells exposed to TA revealed that an aggregation of TA crystals was occurred, which appeared to induced cell death or inhibition around the location, resulting in a clear space between the cells (Figure 3.21.(a)). Contrary to that, TA-NPs were not visually detected by microscopy (Figure 3.21 (b)). The clear spaces were also observed in the cells exposed to TA-NPs but not as much as in the cells exposed to TA.

(a)



(b)

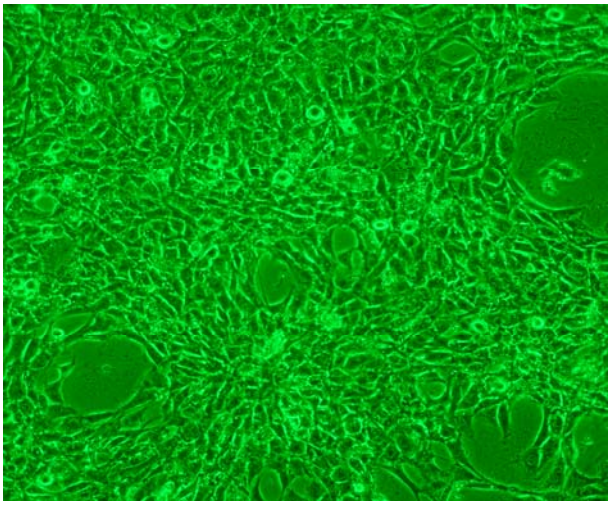
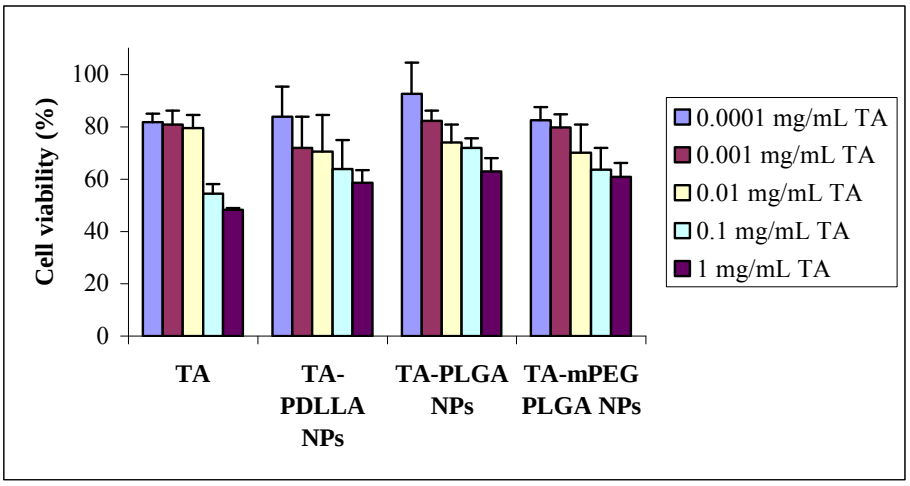


Figure 3.21: Image of BALB/c 3T3 cells after being exposed to TA and TA-NPs. Phase contrast microscopy 33x. Cells were exposed to samples for 24 hours at 37°C. (a) Cells were exposed to TA at a concentration of 1 mg/mL (b) Cells were exposed to TA-NPs containing the same concentration of TA.

(a)



(b)

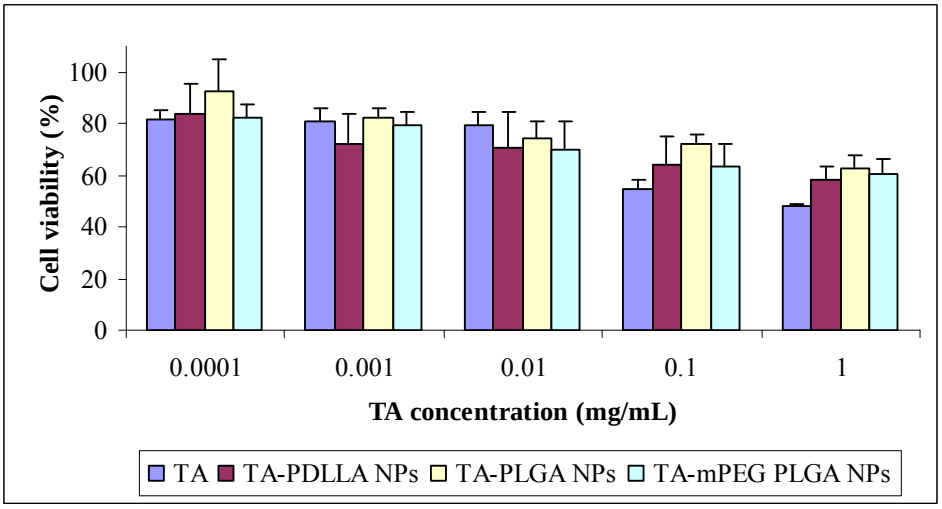


Figure 3.22: Effect of TA and TA-NPs on BALB/c 3T3 cell viability. Cells were seeded at 15,000 cells/well in D10 media and exposed to TA (0.1 µg/mL to 1 mg/mL) and TA-NPs with drug concentration equivalent to TA, for 24 hours. The different chart designs are used to assist in understanding the effects of samples on the BALB/c 3T3 cell viability. (a) Cell viability against type of samples (b) Cell viability against TA concentration (mg/mL). Data presented as mean ± SD (n=3).

The toxicity studies on BALB/c 3T3 cell model demonstrated a dose dependent decrease in cell viability with the increasing sample concentration (Figure 3.22). Although at the TA concentration of 0.1mg/mL and 1 mg/mL the cell viability was dramatically decreased, there was little effect on concentrations from 0.1 µg/mL to 0.01 mg/mL. This indicates that the cells were not susceptible to TA at the

concentration at and below 0.01 mg/mL. Similar results were observed after exposure to all types of NPs at the same concentration of TA (0.1 µg/mL to 0.01 mg/mL).

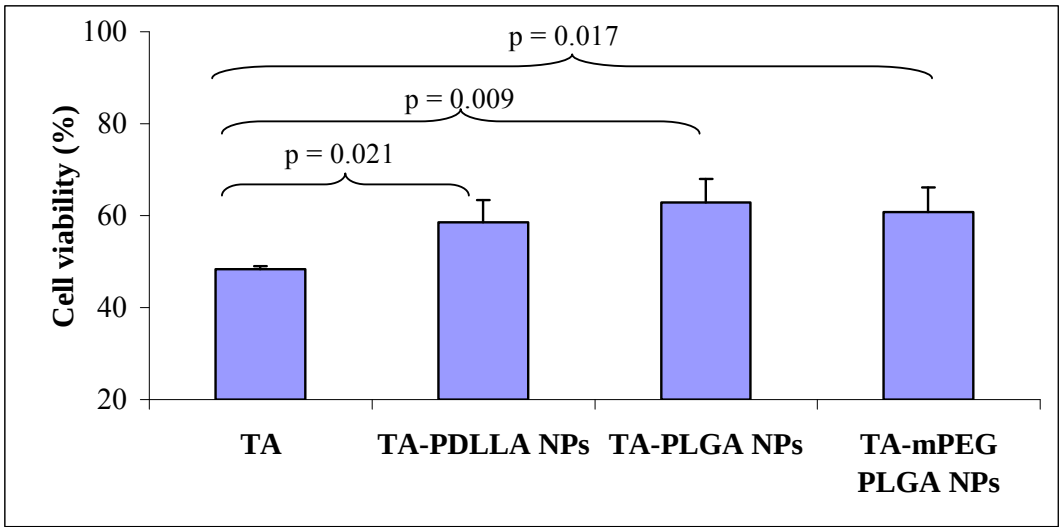


Figure 3.23: Effect of TA (1 mg/mL) and TA-NPs on BALB/c 3T3 cell viability. Cells were seeded at 15,000 cells/well in D10 media and were exposed to 1 mg/mL of either TA or TA-NPs with drug concentration equivalent to TA for 24 hours. Cell viability was determined using MTT assay. Data represent mean ± SD (n=3). The significant difference was considered if $p < 0.05$.

At TA concentration of 1 mg/mL, the differences on the cell viability after being exposed to TA NPs compared to TA control were statistically significant (Figure 3.33) with a reduction of cell viability down to around 50%. Among three types of NPs, TA PLGA NPs appeared to be the most effective in protecting the cells from TA toxicity. Although only around 70% of cells were viable after being exposed to TA-NPs, it demonstrates that the TA NPs show significantly less cell toxicity than TA in this cell model.

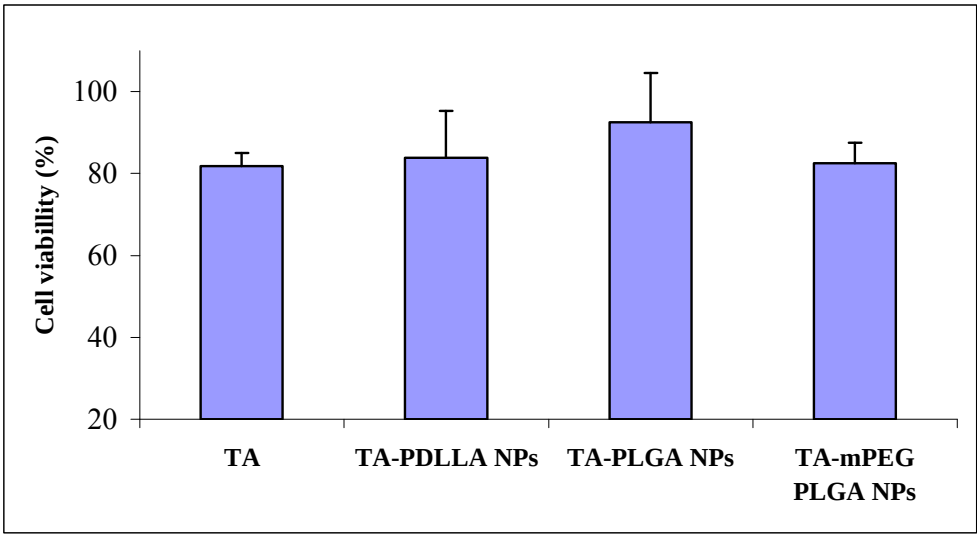


Figure 3.24: Effect of TA (0.1 mg/mL) and TA-NPs on BALB/c 3T3 cell viability. Cells were seeded at 15,000 cells/well in D10 media and exposed to 0.1 mg/mL of either TA or TA-NPs with drug concentration equivalent to TA for 24 hours. Cell viability was determined using MTT assay. Data represent mean ± SD (n=3). The significant difference was considered if $p < 0.05$.

At TA concentration of 0.1 mg/mL, the difference is not statistically significant due to the high levels of standard deviation, resulting from the biological variability between triplicate experiments (Figure 3.34).

The relative contribution of the NPs themselves to toxicity was also investigated (Figure 3.35). The cell viabilities under exposure to TA loaded NPs containing the highest concentration of TA (1 mg/mL) and empty NPs containing an equal amount of NPs as the TA loaded NPs were assessed. The result demonstrates that the difference was not statistically significant, indicating that NPs did not contribute to the TA toxicity on BALB/c 3T3 cell model .

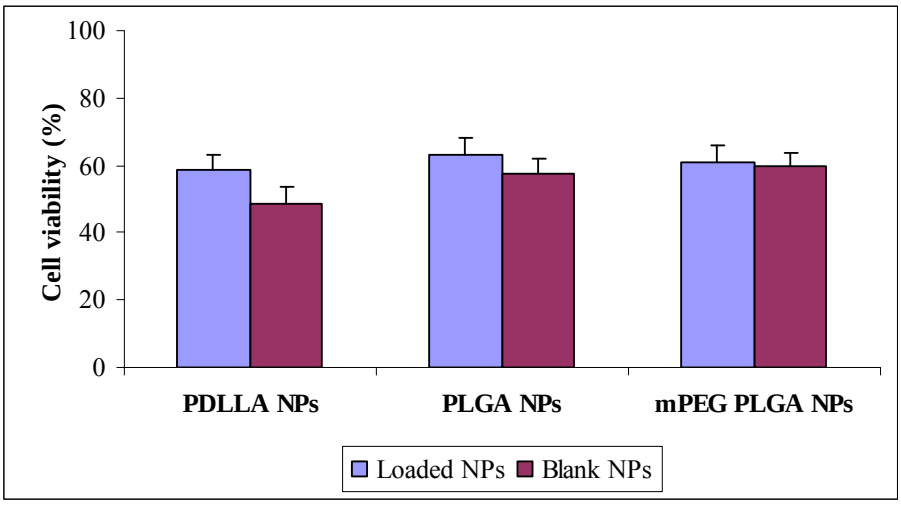


Figure 3.25: Effect of empty NPs and TA-NPs on BALB/c 3T3 cell viability. Cells were seeded at 15,000 cells/well in D10 media and exposed to either TA-NPs with TA concentration of 1 mg/mL or empty NPs with an equivalent amount of NPs to TA-NPs, for 24 hours. Cell viability was determined using MTT assay. Data represent mean ± SD (n=3).

3.3.3 ARPE 19 cell viability assessment

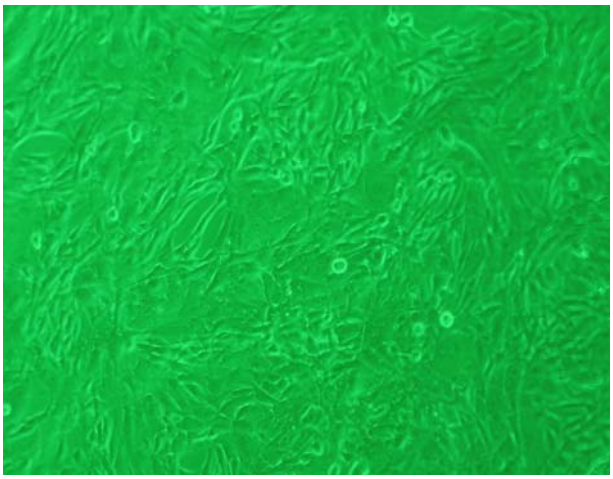


Figure 3.26: Image of ARPE 19 cells. A phase contrast microscopy at 33x magnification. The cells were inoculated in DF12 media (80% confluence).

Recently, TA has been extensively investigated in the treatment of retinal diseases [84-86, 91]. Since TA-NPs are potential carriers to treat retinal diseases, it was imperative to test TA-NPs toxicity against a more specific and relevant cell model. TA has been

investigated for toxicity using ARPE 19 cells [97, 98, 110], therefore the non malignant retinal epithelial cell-line, ARPE 19, was used for this purpose.

To assess the toxicity of TA and TA-NPs, *in vitro* toxicity studies were also conducted by exposing ARPE 19 cell culture to TA and TA-NPs over 24 hours at 37°C. The range of TA concentration was similar to those applied on BALB/c 3T3 cell viability assessment based on the previous toxicity study of TA conducted by Yeung [98].

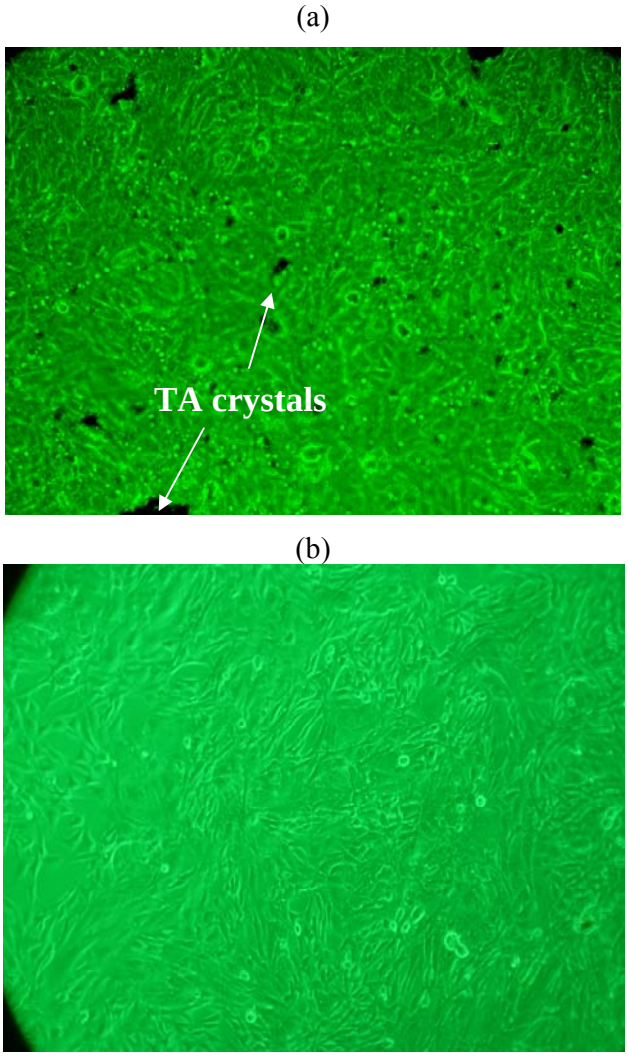
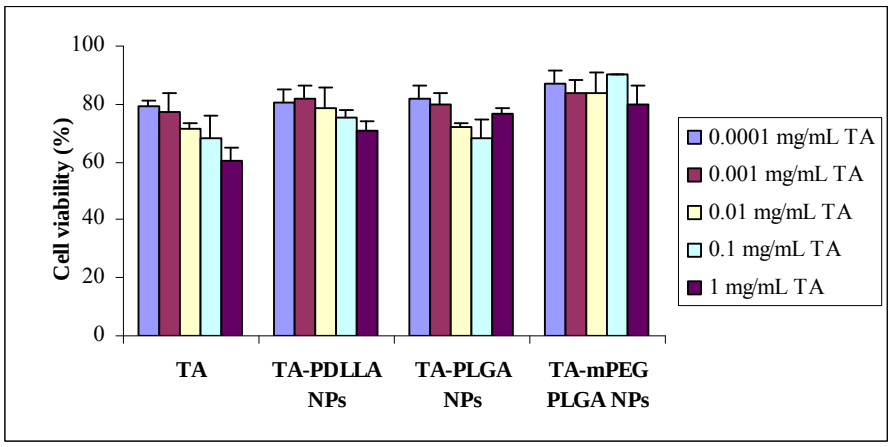


Figure 3.27: Morphology of ARPE 19 cells after being exposed to TA and TA-NPs. Phase contrast microscopy at 33x magnification. Cells were exposed to samples for 24 hours at 37°C. (a) Cells were exposed to TA at a concentration of 1 mg/mL (b) Cells were exposed to TA-NPs containing the same concentration of TA.

Phase contrast microscopy was also used to observe the cells exposed to TA and TA-NPs. An aggregation of TA crystal was observed on the cells exposed to TA, however, the cell morphology appeared to be the same after exposure (Figure 3.37 (a)). The TA NPs were not detected by phase contrast microscopy due to sub-micron size of the NPs (Figure 3.37 (b)). Due to time limitation, investigation on morphological alteration after exposure on the cells was not conducted.

(a)



(b)

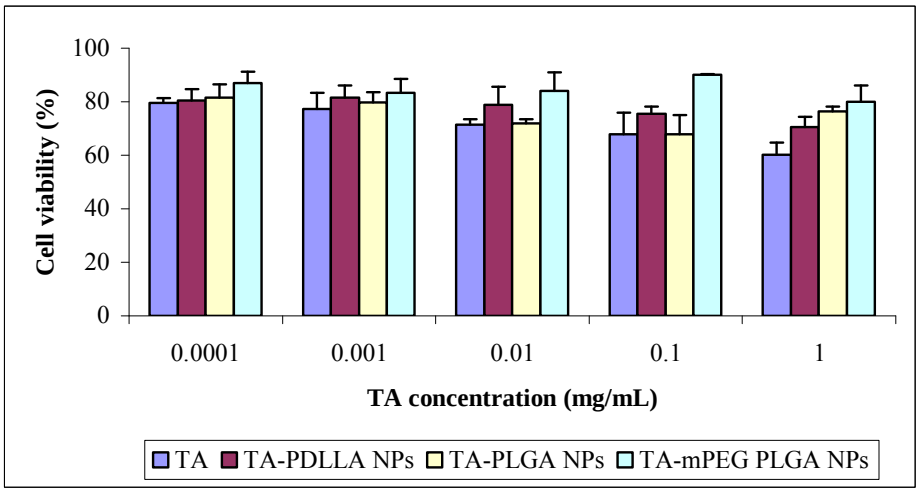


Figure 3.28: Effect of TA and TA-NPs on ARPE 19 cell viability. Cells were seeded at 20,000 cells/well in DF12 media and exposed to TA (0.1 µg/mL to 1 mg/mL) and TA-NPs with drug concentration equivalent to TA, for 24 hours. The different chart designs are used to assist in understanding the effects of samples on the BALB/c 3T3 cell viability. (a) Cell viability against type of samples (b) Cell viability against TA concentration (mg/mL). Data presented as mean (n=3) ± SD.

High toxicity of TA after 24 hour exposure to ARPE 19 was observed at the highest concentration (1 mg/mL) (Figure 3.38 (a)). It reduced the cell viability down to 60%. The differences in cell viability after 24 hour exposure to TA and TA-NPs, at the TA concentration of 1 mg/mL, were statistically significant (Figure 3.39). At concentration of 0.1 mg/mL or less, the TA was still found to be relatively toxic compared to TA-NPs. However, regarding TA-NPs, around 80-100% viable cells were observed on TA NPs with TA concentration at 0.1 mg/mL or less, suggesting that the TA NPs did not induce toxicity on those concentration.

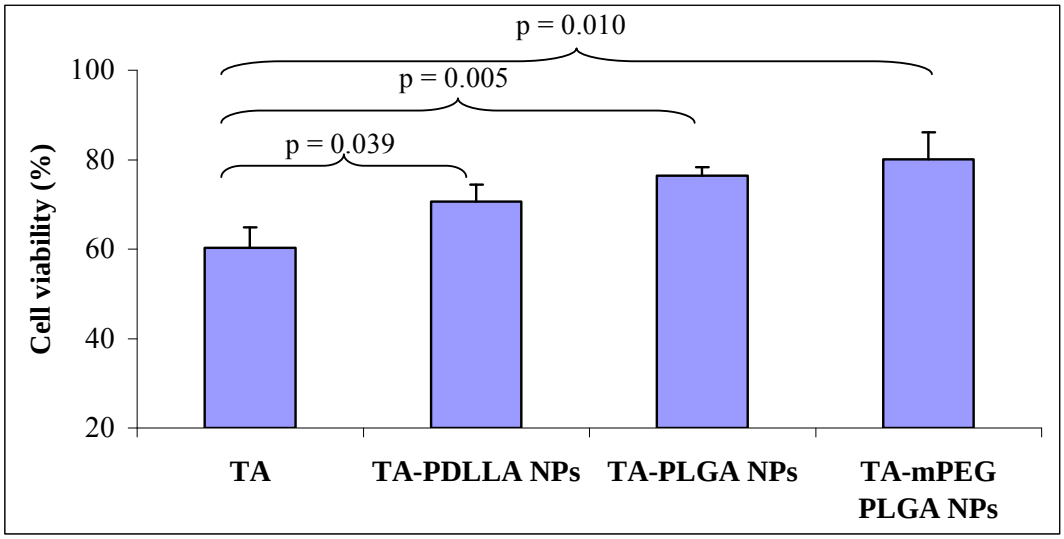


Figure 3.29: Effect of TA (1 mg/mL) and TA-NPs on ARPE 19 cell viability. Cells were seeded at 20,000 cells/well in DF12 media and exposed to 1 mg/mL of either TA or TA-NPs with drug concentration equivalent to TA for 24 hours. Cell viability was determined using MTT assay. Data represent mean ± SD (n=3).

The toxicity of all TA NPs were significantly less than TA alone in ARPE 19 cells. Although only around 80% cells were viable after 24 hours exposure, NPs did not appear to induce toxicity.

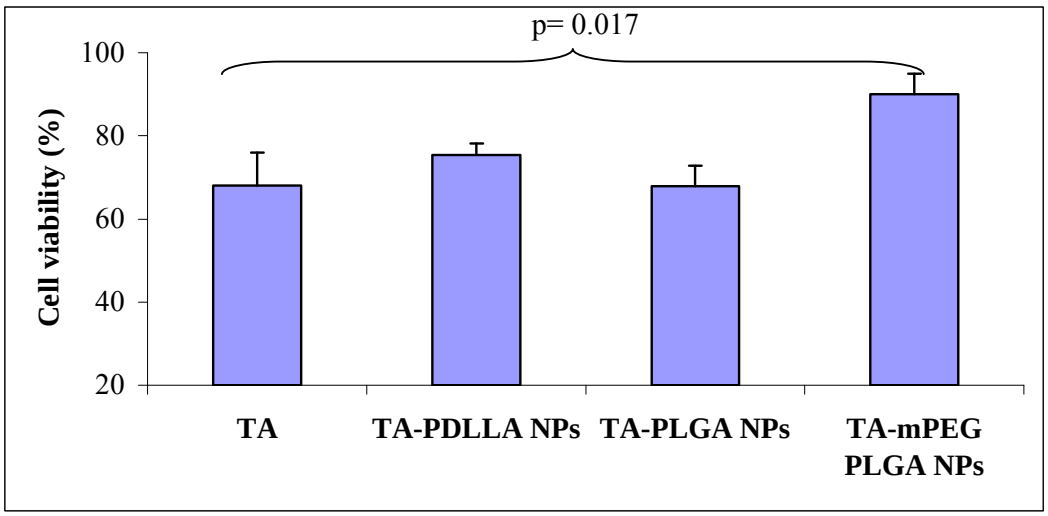


Figure 3.30: Effect of TA (0.1 mg/mL) and TA-Nps on ARPE 19 cell viability. Cells were seeded at 20,000 cells/well in DF12 media and exposed to 0.1 mg/mL of either TA or TA-NPs with drug concentration equivalent to TA for 24 hours. Cell viability was determined using MTT assay. Data represent mean ± SD (n=3).

Among three type of TA-NPs, TA-mPEG PLGA NPs was apparently the least toxic. The similar trend is also observed at the TA concentration of 0.1 mg/mL (Figure 3.40), in which TA-mPEG PLGA NPs appeared to be the safest NPs. From the same figure, we revealed that the differences of cell viability after exposure to TA-PDLLA NPs and TA-PLGA NPs were statistically insignificant compared to cell viability after TA exposure.

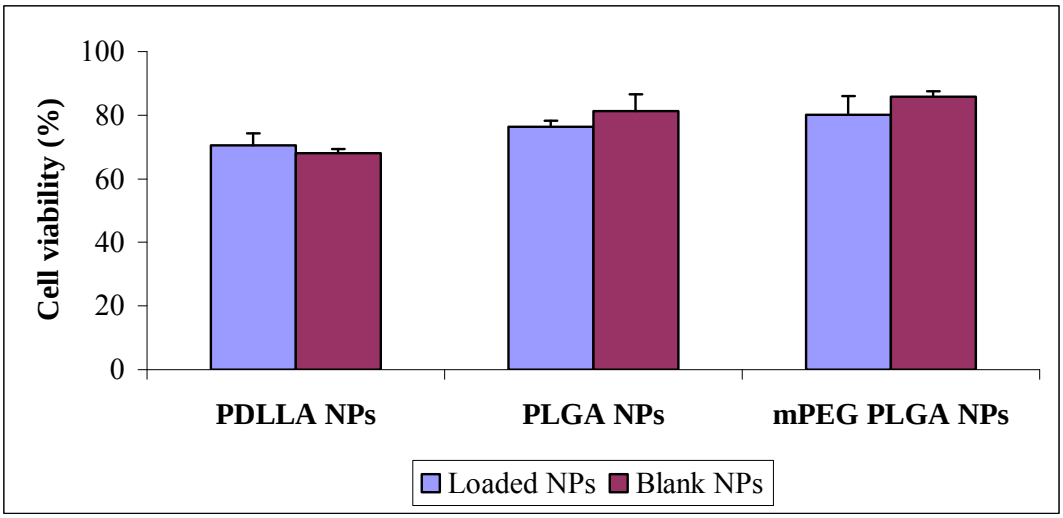


Figure 3.31: Effect of empty NPs and TA-NPs on ARPE 19 cell viability. Cells were seeded at 20,000 cells/well in DF12 media and exposed to either TA-NPs with TA concentration of 1 mg/mL or empty NPs, for 24 hours. Cell viability was determined using MTT assay. Data represent mean ± SD (n=3).

The toxicity of NP themselves were also assessed using ARPE 19. In comparison of the cell viability exposed to TA-NPs (which contain TA at 1 mg/mL) and empty NPs, it was revealed that there were no statistically significant differences between empty and TA-NPs (Figure 3.41).

To summarise, TA was found to be toxic at the highest concentration (1 mg/mL) on both cell models. Significant reduction of cell viability of cells was obtained in BALB/c 3T3 (down to 50%) and ARPE 19 (down to 60%). This finding is in agreement with other TA toxicity studies on ARPE 19 cell model [98, 110]. The high toxicity level of TA on the highest concentration (1 mg/mL) was speculated to be due to substantially large particle size compared to the NPs. Szurman et al [111] demonstrated that the cytotoxic effect of TA was governed by sedimented TA crystals or aggregates in direct contact with the cell surface. In addition to the results, BALB/c 3T3 cell viability reduction was found to be dose-dependent. They are also more sensitive to TA and TA NPs than ARPE 19.

All types of TA-NPs were significantly less toxic than TA at the TA concentration of 1 mg/mL on both cell models. The levels of cell viability after exposure to TA-NPs

were higher than those after exposure of TA. The differences of cell viability exposed to TA and TA-NPs were statistically significant ($p<0.05$). In addition, empty NPs and TA-NPs show the similar toxicity level, indicating that the cell viability of both cell culture models was less affected by NPs themselves. These findings suggest that the delivery of TA by NPs may potentially reduce the potential side effect of TA. Bejjani et al [112] demonstrated that PLGA NPs was considered to be non toxic upon RPE and its rapid internalization enables gene transfer and expression in RPE cells.

GENERAL DISCUSSION

NP delivery was investigated for TA since it has potential advantages of providing controlled release and prolonging stability of TA. Moreover, due to their sub-micron size, NPs are more acceptable than larger particles for intra vascular administration as they are small enough for cellular internalization.

TA was selected as a model for hydrophobic drugs for studying polymeric NP formulations. The control of particle size of hydrophobic drugs appears to be problematic in parenteral administration, since the hydrophobic particles tend to be physically unstable and aggregate. Polymeric NP formulations may provide alternatives to overcome these problems as surface of polymeric NPs can be readily modified.

This study involves investigation of three types of polymers for incorporation of TA into NPs: poly (D,L, lactide) (PDLLA), poly (D,L, lactide-co-glycolide) (PLGA) and methoxy polyethyleneglycol-poly (D,L, lactide-co-glycolide) (mPEG-PLGA). PLGA is more hydrophilic than PDLLA due to the presence of glycolic acid on the copolymerisation of lactic acid with glycolic acid. The hydrophilicity in mPEG PLGA is further enhanced by an addition of polyethyleneglycol group. The hydrophobicity level of those polymers follows this order: PDLLA > PLGA > mPEG-PLGA.

4.1 Polymeric NP formulations for TA delivery

Preparation of polymeric NPs was carried out by either the solvent diffusion or solvent evaporation method. The former method has an advantage of producing sub-micron particles, using non toxic organic solvents, in an effective and reproducible manner [12, 47-49, 57, 58, 113]. The latter method is commonly used to manufacture microparticles [15, 114]. However, by appropriate modification NPs can also be easily made by this method. One of the advantages of applying this method is that it provides high levels of yield, loading and entrapment efficiency, especially for hydrophobic drugs due to the slower evaporation of the organic solvent, which

results in slower precipitation of NPs. The difference which can be distinguished for both methods is the application of different solvent system. The solvent diffusion method involved acetone as the organic solvent, whereas in the solvent evaporation method, DCM which is immiscible with water, was employed instead of acetone. The solvent system in this study played an important role in determining the characteristics of NPs.

The NPs were characterized for their particle size, surface charge, yield and loading value, and TA entrapment efficiency. An i*n vitro* release study was conducted to investigate the release characteristics of all types of TA-NPs compared to TA control (free TA).

The empty NPs made by both methods show excellent characteristics. Both methods can provide sub-micron particles with those made by the solvent diffusion method being smaller than those made by solvent evaporation. In terms of surface charge, the zeta potential of those particles was highly negative, indicating that NPs were well-dispersed in the medium. In a comparison among polymers, PDLLA, which is the most hydrophobic polymer, exhibited the largest particle size, and the lowest yield. In the solvent diffusion method, this is due to a rapid diffusion of organic solvent into the aqueous stabiliser phase, leaving the polymer with strong hydrophobic bonds to associate, leading to aggregation and the formation of microparticles during NP preparation, which were not expected. Low concentration of the stabiliser in the solvent diffusion method (0.6% PVA) may have failed to effectively stabilise the emulsion, resulting in aggregation of polymer.

Based on those characteristics, an attempt to incorporate 40 mg TA into 200 mg polymer was made using either solvent diffusion or solvent evaporation. To determine the concentration of TA, either for loading determination or release study, an HPLC method reported by Gupta[74] was adopted with some modification to optimize the separation of the peaks. This method was selected since it provides a stability indicating assay for TA. Validation of HPLC assay was conducted in order to develop an accurate and reproducible assay to determine TA concentration. The main parameters of validation such as: linearity of detector response, system precision, recovery (accuracy), sensitivity and selectivity, were examined. The

investigation revealed that the validated modified HPLC method developed in this study was suitable for the determination TA in the formulation and release experiments.

Initially, solvent diffusion method was employed to prepare TA-loaded NPs with theoretical loading of 16.7%. The preparation of TA-PDLLA NPs, the entrapment efficiency initially appeared to be satisfactory (approximately 65%). However, further investigation showed that the NPs obtained were in a mixture of TA-NPs and TA crystals. An optimisation of the method therefore was conducted. By settling down the TA crystals for 30 minutes prior to PVA purification, the crystals were separated and removed from the NPs. It was found that by optimised solvent diffusion method, the yield, loading and entrapment efficiency of TA-NPs were extremely low. This was probably due to the high solubility of TA in acetone, as when acetone diffused out rapidly to the aqueous phase, TA also diffused out from the organic phase.

The solvent evaporation method was then employed to prepare TA-NPs. In using this method, although the size was slightly increased, the yield, loading and entrapment efficiency of NPs made from three different polymers were obtained in reasonably high levels. The increased size was due to the primary droplet formed in the emulsification, which was affected by the concentration of polymer in organic solvent (the viscosity of organic phase). The more viscous the organic phase, the larger the droplet. This is due to the less ability of the viscous organic phase to disperse. Eventually, larger droplet will produce larger NPs [96, 115]. Slow evaporation of DCM also contributed to the formation of large particle size of NPs. However, the use of a sonicator during emulsification effectively reduce the size of NPs[116].

Görner et al[115] demonstrated that an increase in size of the NPs would increase the entrapment efficiency. However, the initial amount of drug added plays an important role in determining the entrapment efficiency of NP preparation. In our study, although the size of TA-mPEG PLGA NPs (around 295 nm) was smaller than that of TA-PLGA NPs (around 325 nm), both TA-PLGA and TA-mPEG PLGA NPs exhibited high entrapment efficiency of around 65%. However, TA PDLLA NPs exhibited the largest size (around 360 nm) and the lowest entrapment efficiency

(30%). The low entrapment efficiency of TA-PDLLA NPs was probably due to the partitioning of polymer and TA between the dispersed and the dispersing phase. The partition is not only to the organic solvent as both polymer and drug molecule may also diffuse across the interface from organic phase to the external phase during NP formation [30].

The preparation of NPs in this study involves PVA as a stabiliser. Although PVA is reported as an effective stabiliser, which can assist to produce NPs with reduced size and high entrapment efficiency [36, 104], it is also reported by other investigator that PVA is a suspected carcinogen [105]. Therefore, purification of NPs was carried out to remove the PVA. High speed centrifugation was selected as the purification technique due to its efficiency to separate the NPs from the supernatant containing PVA. Although high speed centrifugation offers satisfactory results in removing the PVA (Table 3.10 and Figure 3.11), PVA still could not be 100% removed from the system. This finding corresponds with the study reported by Murakami et al [48, 106] which revealed that small amount of PVA appeared to be attached on the surface of NPs as a result of hydrophobic bonding.

Physical stability of TA in NPs was also examined. TA was extracted from TA-NPs after 96 hour-*in vitro* release study, and the extraction samples were injected into the HPLC. A typical chromatogram showed that no degradation product of TA was detected in the extraction (Figure 3.16). This finding was the same for all types of TA-NPs and suggests that TA was fully isolated by NPs from environmental hazards.

In vitro release study was carried out only for the TA-NPs made by solvent evaporation, prone to small entrapment efficiency achieved by TA-NPs made by solvent diffusion. The release characteristics of three types of NPs show biphasic patterns. Initially, a fast constant release was observed over 8 hours (around 20% TA was released), followed by a slow release over 96 hours. The fast release of TA over 8 hours was due to poor entrapment of TA resulting in reduced surface area. The slow release of TA after 24 hours might be performed by TA entrapped in the NPs.
Of the three types of NPs, the greatest release was observed on TA-mPEG PLGA NPs, which were the smallest among those NPs (around 295 nm). Avgoustakis et al [53] demonstrated that the release of drug from mPEG PLGA NPs

was related to the mPEG content, it increases with an increase of mPEG content. The release characteristics of the NPs were normally governed by drug diffusion and polymer erosion, which is a result of polymer degradation. Porosity of the particles, as a result of solvent evaporation during initial preparation may also affect the release behaviour. The more porous the NPs, the higher the drug diffusion rate would be . Hans and Lowman [12] reported that large particles would exhibit longer sustained release than small ones. They also illustrated that in terms of loading, the higher the loading may cause an increase in the release rate. From the investigation, it was found that TA- mPEG-PLGA with the highest loading (67%) show the fastest release (around 90% of TA released over 96 hours), while TA-PDLLA NPs with the particle sized around 360 nm, showed the slowest release among three types of NPs.

Limitation of the formulation study

There are several limitations in this study. Both methods used in the TA-NPs preparation could not produce NPs with size less than 200 nm. This is probably due to the relatively high polymer concentration in the organic phase. In terms of loading, the theoretical loading was too high (16.67%) to be loaded into the NPs resulting in very low entrapment efficiency and yield.

4.2. *In vitro* toxicity study of TA-loaded NPs

To assure the safety of polymeric NP formulations for TA delivery, it is essential that the toxicities of TA-NPs are assessed. Basic toxicity assessment of TA-NPs was carried out in this study upon two different cell lines: BALB/c 3T3 (fibroblasts) cells which are the immortalised and non malignant cells, and ARPE 19 (human retinal pigment epithelia) cells, which represent retinal cells. The toxicity was represented by percentage of cell viability of the samples over the control.

In our study, we revealed that TA at 1 mg/mL was toxic. It reduced the cell viability of BALB/c 3T3 cell model down to 50% and ARPE 19 cell model down to 60%. However the TA-NPs containing the same amount of TA were less toxic than TA. In both cell models, it was found that BALB/c 3T3 cell model was more sensitive than ARPE 19. It also showed dose-dependent reduction in exposure to TA.

Although the effect of particle size of either TA or TA-NPs on the cell viability did not particularly investigated due to time limitation, the distinct difference of size between TA (microsize) and TA-NPs (nanosize) can be one of the reasons to explain the high toxicity of TA in this study. The possibility of TA crystals to be aggregated also contributed to the high potential of being toxic.

Limitation of the in vitro toxicity study

The MTT assay is an easy and simple assay and it is the best to be used with cells which show high mitochondrial activity. Cells with low mitochondrial activity may show low intensity leading to potential assay error.

CONCLUSION AND SCOPE FOR FUTURE WORK

5.1 Conclusion

The present study demonstrated that incorporation of TA into NPs with favourable characteristics can be achieved by solvent diffusion and solvent evaporation method using PDLLA, PLGA and mPEG PLGA. Although solvent diffusion produced NPs sized around 200 nm, which was less than of those made by solvent evaporation, solvent evaporation was superior to solvent diffusion method in terms of yield, loading and entrapment efficiency. In terms of NP characterization, TA NPs synthesized from mPEG PLGA exhibited the smallest particle size and the highest entrapment efficiency whereas PDLLA was more likely to produce particles with large size and low entrapment efficiency.

All types of NPs studied demonstrated sustained release ability to certain degrees. This was particularly evident as the amount of the drug released reduced after 24 hours and remained constant subsequently. TA-mPEG NPs, which has the highest drug loading exhibited the fastest release, while TA-PDLLA NPs appeared to release TA in the slowest rate.

Stability of TA in NPs was found to be superior to that in drug suspension. Undetected degradation products in TA-NPs over 96 hours, indicated that the stability of TA has been enhanced by NP formulations.

An *in vitro* toxicity study revealed that in both BALB/c 3T3 and ARPE 19 cells, TA was toxic on the highest concentration (1 mg/mL) while the TA-NPs containing TA at the same concentration, were less likely to be toxic. The difference of cell viability determined under exposure of either TA loaded- NPs or blank NPs was found to be statistically insignificant ($p>0.05$), indicating that the components of NPs beside the drug did not induce toxicity. BALB/c 3T3 cells were more sensitive than ARPE 19 to TA and TA-NPs.

In summary, the TA NPs which were made from biodegradable and biocompatible polymers, have shown a promising delivery for TA, in which NPs provide controlled release properties, are potential to reduce the toxicity of TA, and are able to prolong the stability of TA.

5.2 Scope for future work

Although TA loaded NPs were successfully made by a simple solvent evaporation method, the ideal manufacture of NPs has not been achieved due to the use of a toxic organic solvent and a potentially toxic stabiliser. The method conducted in this study, failed to produce the NPs with size less than 200 nm. It will be worthwhile to investigate the preparation parameters of NPs which can produce small NPs (< 200 nm) and can be potentially scaled up with high drug loading and yield. The polymer which has a nature like mPEG-PLGA is considered to be a suitable polymer for NP manufacture since it is biocompatible and able to prolong the circulation of NPs.

The mechanism of release study of TA-NPs needs to be investigated further. The experimental conditions for the *in vitro* release study should be further optimised to ensure sink conditions in the system. As for the formulation, an additional feature which can minimize burst release should be taken into consideration in the design of the formulations.

The properties to be available for cellular uptake have become focus to explore in the preparation of NPs. Since TA-NPs considered to be less toxic than TA itself, it will be worthwhile to study the mechanism of cellular traffic of TA-NPs to understand the distribution and interaction of NPs with the biological system.

REFERENCES

1. Robinson J, editor. Sustained and controlled release drug delivery systems. New York: Marcell Dekker; 1978.
2. Svenson S, editor. Polymeric drug delivery I: particulate drug carriers. Washington DC: American Chemical Society; 2005.
3. Linhardt RJ. Biodegradable polymers for controlled release of drugs. In: Rossof M, editor. Controlled Release of Drugs: Polymers and aggregate systems. New York: VCH Publishers, Inc; 1989. p. 53-95.
4. Juliano RL, editor. Drug delivery systems, characteristics and biomedical applications. New York: Oxford University Press, Inc.; 1980.
5. Schwenderman AP, Cardamone M, Klibanov A, Langer R. Stabillity of protein and their delivery from biodegradable polymer microspheres. In: Cohen H, Bernstein H, editors. Microparticulate systems for the delivery of proteins and vaccines. New York: Marcell Dekker, Inc; 1996.
6. Park K, editor. Controlled drug delivery, challenges and strategies. Washington, DC: American Chemical Society; 1997.
7. Kreuter J. Colloidal drug delivery. In. New York: Marcell Dekker; 1994.
8. Kreuter J. Nanoparticles. In: Kreuter J, editor. Colloidal drug delivery systems. New York: Marcel Dekker; 1994.
9. Reis CP, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. Nanomedicine: Nanotechnology, Biology, and Medicine 2006;2:8-21.
10. Pison U, Welte T, Giersig , Groneberg D.A. Nanomedicine for respiratory diseases. Eur J Pharm Sci 2006;533:341-350.
11. Labhasetwar V, Song C, Levy RJ. Nanoparticle drug delivery system for restenosis. Adv Drug Del Rev 1997;24(1):63-85.
12. Hans ML, Lowman AM. Biodegradable nanoparticles for drug delivery and targeting. Current Opinion in Solid State and Materials Science 2002;6(4):319-327.
13. Olivier JC. Drug transport to brain with targeted nanoparticles. NeuroRx 2005;2(1):108-19.

14. Reis CP, Neufeld RJ, Ribeiro AJ, Veiga F. Nanoencapsulation II. Biomedical applications and current status of peptide and protein nanoparticulate delivery systems. Nanomedicine: Nanotechnology, Biology, and Medicine 2006;2:53-65.

15. Guiot P, Couvreur P, editors. Polymeric nanoparticles and microspheres. Boca Raton: CRC Press; 1986.

16. Kataoka K. Targeted polymeric drugs. In: Park K, editor. Controlled drug delivery, Challenges and Strategy. Washington DC: ACS Professional Reference Book; 1997. p. 49-71.

17. Oppenheim R. Nanoparticles. In: Juliano RL, editor. Drug delivery systems, characteristics and biomedical applications. New York: Oxford University Press; 1980. p. 177.

18. Domb A, editor. Polymeric site specific pharmacotherapy. New York: John Wiley and Sons; 1994.

19. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJA. Nanotoxicology. Occup Environ Med 2004;61:727-728.

20. Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J Control Release 1999;57(2):171-85.

21. des Rieux A, Fievez V, Garinot M, Schneider YJ, Preat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. J Control Release 2006;116(1):1-27.

22. Kirchweger G. Nanoparticles-the next big thing? [Commentary]. The American Society of Gene Therapy; 2002 [cited 13 Agust 2005]. Available from: www.idealibrary.com.

23. Ticho U, Blumenthal M, Zonis S, Gal A, Blank I, Mazor ZW. A clinical trial with Piloplex- a new long acting pilocarpine compound: preliminary report. Ann. Ophthalmol 1979;4:555-561.

24. Calvo P, Martinez J, Lopez MI, Calonge M, Pastor JC, Alonso MJ. Polyester nanocapsules as new topical oclar delivery systems for cyclosporin. Pharm Res 1996;13:311-315.

25. Campos AM, Sanchez A, Alonso MJ. Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A. Int J Pharm 2001;224:159-168.

26. Gelperina SE, Khalansky AS, Skidan IN, Smirnova ZS, Bobruskin AI, Severin SE, et al. Toxicological studies of doxorubicin bound to polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles in healthy rats and rats with intracranial glioblastoma. Toxicol Lett 2002;126(2):131-141.

27. Mu L, Feng SS. A novel controlled release formulation for the anticancer drug paclitaxel (Taxol®): PLGA nanoparticles containing vitamin E TPGS. J Control Rel 2003;86(1):33-48.

28. Dong Y, Feng SS. Nanoparticles of poly(D,L-lactide)/methoxy poly(ethylene glycol)-poly(D,L-lactide) blends for controlled release of paclitaxel. J Biomed Mater Res A 2006;78(1):12-9.

29. Fonseca C, Simoes S, Gaspar R. Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity. J Control Rel 2002;83(2):273-286.

30. Mu L, Feng SS. Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol®). J Control Rel 2002;80(1-3):129-144.

31. Mukherjee P, Bhattacharya R, Wang P, Wang L, Basu S, Nagy JA, et al. Antiangiogenic properties of gold nanoparticles. Clin Cancer Res 2005;11(9):3530-4.

32. Hood JD. Tumor regression by targeted gene delivery to the neovasculature. Science 2002;296:2404-2407.

33. Singh J, Pandit S, Bramwell VW, Alpar HO. Diphtheria toxoid loaded poly-(epsilon-caprolactone) nanoparticles as mucosal vaccine delivery systems. Methods 2006;38(2):96-105.

34. Diwan M, Elamanchili P, Cao M, Samuel J. Dose sparing of CpG oligodeoxynucleotide vaccine adjuvants by nanoparticle delivery. Curr Drug Deliv 2004;1(4):405-12.

35. Ranade V, Hollinger M. Drug delivery systems. Second ed. New York: CRC Press Inc.; 2004. p. 226-229.

36. Scholes PD, Coombes AGA, Illum L. The preparation of sub-200 nm poly (lactide-co-glycolide) microspheres for site specific drug delivery. J Control Rel 1993;25:145-153.

37. Yolles S, Sartori MR. Degradable polymers for sustained release. In: Juliano RL, editor. Drug Delivery Systems. New York: Oxford University Press; 1980.

38. Chawla JS, Amiji MM. Biodegradable poly (ε–caprolactone) nanoparticles for tumor-targeted delivery of tamoxifen. Int J Pharm 2002;249:127-138.

39. Chew JL, Wolfowicz CB, Mao HQ, Leong KW, Chua KY. Chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen, Der p 1 for oral vaccination in mice. Vaccine 2003;21(21-22):2720-9.

40. Illum L, Jabbal-Gill I, Hinchcliffe M, Fisher AN, Davis SS. Chitosan as a novel nasal delivery system for vaccines. Adv Drug Del Rev 2001;51(1-3):81-96.

41. van der Lubben IM, Verhoef JC, Borchard G, Junginger HE. Chitosan for mucosal vaccination. Adv Drug Del Rev 2001;52(2):139-44.

42. Thanou M, Verhoef J, Junginger H. Oral drug absorption enhancement by chitosan and its derivatives. Adv Drug Del Rev 2001;52(2):117-126.

43. Scholes PD, Coombes AGA, Davies MC, Illum L, Davis SS. Particle engineering of Biodegradable Colloids for site-specific drug delivery. In: Park K, editor. Controlled drug delivery, Challenges and strategies. Washington DC: American Chemical Society; 1997.

44. Ignatius AA, Claes LE. In vitro biocompatibility of bioresorbable polymers: poly(L, DL-lactide) and poly(L-lactide-co-glycolide). Biomaterials 1996;17(8):831-9.

45. Perrin DE, J.P E. Polyglycolide and polylactide. In: Domb A, Kost J, Wiseman DM, editors. Handbook of biodegradable polymers. Amsterdam: Harwood academic publishers; 1997. p. 3-26.

46. Lewis D. Controlled release of bioactive agents from lactide/glycolide polymers. In: Chasin M, Langer R, editors. Biodegradable polymers as drug delivery systems. New York: Marcell Dekker; 1990. p. 1-41.

47. Chorny M, Fishbein I, Danenberg HD, Golomb G. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. J Control Rel 2002;83(3):389-400.

48. Murakami H, Kobayashi M, Takeuchi H, Kawashima Y. Preparation of poly(-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method. Int J Pharm 1999;187(2):143-152.

49. Murakami H, Kobayashi M, Takeuchi H, Kawashima Y. Further application of a modified spontaneous emulsification solvent diffusion method to various types of PLGA and PLA polymers for preparation of nanoparticles. Powder Technology 2000;107(1-2):137-143.

50. Liu M, Dong J, Yang Y, Yang X, Xu H. Characterization and release of triptolide-loaded poly (D,L-lactic acid) nanoparticles. Euro Pol J 2005;41(2):375-382.

51. Gaspar M, Blanco D, Cruz MEM, Alonso MJ. Formulation of L-asparaginase-loaded poly(lactide-co-glycolide) nanoparticles: influence of polymer properties on enzyme loading, activity and in vitro release. J Control Rel 1998;52:53-62.

52. Avgoustakis K. Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: preparation, properties and possible applications in drug delivery. Curr Drug Deliv 2004;1(4):321-33.

53. Avgoustakis K, Beletsi A, Panagi Z, Klepetsanis P, Karydas AG, Ithakissios DS. PLGA-mPEG nanoparticles of cisplatin: in vitro nanoparticle degradation, in vitro drug release and in vivo drug residence in blood properties. J Control Rel 2002;79(1-3):123-135.

54. Avgoustakis K, Beletsi A, Panagi Z, Klepetsanis P, Livaniou E, Evangelatos G, et al. Effect of copolymer composition on the physicochemical characteristics, in vitro stability, and biodistribution of PLGA-mPEG nanoparticles. Int J Pharm 2003;259(1-2):115-127.

55. Jiao J, Burgess DJ. Characterisation and analysis of dispersed systems. In: Burgess DJ, editor. Injectable disperse systems; formulation, processing and performance. Boca Raton: Taylor & Francis Group; 2005.

56. Mainardes RM, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. Int J Pharm 2005;290(1-2):137-144.

57. Kwon H-Y, Lee J-Y, Choi S-W, Jang Y, Kim J-H. Preparation of PLGA nanoparticles containing estrogen by emulsification-diffusion method. J Colloids and Surfaces A: Physicochemical and Engineering Aspects 2001;182(1-3):123-130.

58. Choi S-W, Kwon H-Y, Kim W-S, Kim J-H. Thermodynamic parameters on poly(D,L-lactide-co-glcolide) particle size in emulsification-diffusion process. Journal of Colloid and Surfaces 2002;201:283-289.

59. Sahoo SK, Panyam J, Prabha S, Labhasetwar V. Residual polyvinyl alcohol associated with poly (,-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Rel 2002;82(1):105-114.

60. Frenkel OH, Debotton N, Benita S, Altschuler Y. Targeting of nanoparticles to the clathrin-mediatic endocytic pathway. Biochem Biophysic Res Comm 2007;353:26-32.

61. Maulding H. Prolonged delivery of peptide by macrocapsules. In: Anderson JM, Kim SW, editors. Advances in Drug Delivery Systems. Amsterdam: Elsevier; 1987. p. 170-171.

62. Dalwadi G, Benson HA, Chen Y. Comparison of diafiltration and tangential flow filtration for purification of nanoparticle suspensions. Pharm Res 2005;22(12):2152-62.

63. Freshney R. Culture of animal cells, a manual of basic technique. second edition ed. New York: Alan R. Liss,Inc.,; 1987. p. 1-4;127-129;245.

64. Hornof M, Toropainen E, Urtti A. Cell culture models of the ocular barriers. Eur J Pharm Biopharm 2005;In Press, Corrected Proof.

65. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1-2):55-63.

66. Aaronson SA, Todaro GJ. Development of 3T3-like lines from Balb-c mouse embryo cultures: transformation susceptibility to SV40. J Cell Physiol 1968;72:141-148.

67. Popiolkiewicz J, Polkowski K, Skierski JS, Mazurek AP. In vitro toxicity evaluation in the development of new anticancer drugs-genistein glycosides. Cancer Lett 2005;229:67-75.

68. Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, A Human Retinal Pigment Epithelial Cell Line with Differentiated Properties. Exp Eye Res 1996;62(2):155-170.

69. Foye W, Lemke T, Williams D. Principles of medicinal chemistry. Fourth ed. Baltimore: Williams & Wilkins; 1995.

70. Derendorf H, Rohdewald P, Hochhaus G, Mollmann H. HPLC determination of glucocorticoid alcohols, their phosphates and hydrocortisone in aqueous solutions and biological fluids. J Pharm Biomed Anal 1986;4(2):197-206.

71. Dollery C. Therapeutic drugs. Second ed. Edinburgh: Churchill Livingstone; 1999.

72. Sieh DH. Triamcinolone acetonide. In: Florey K, editor. Analytical profiles of drug substances. New Jersey: Academic Press; 1982. p. 619-623.

73. Reynolds J, editor. Martindale, the extra pharmacopeia. 28th ed ed. London: The Pharmaceutical Press; 1982.

74. Gupta V. Stability of triamcinolone acetonide solution as determined by high performance liquid chromatography. J Pharm Sci 1982;72(12):1453-1456.

75. Timmins P, Gray EA. The degradation of triamcinolone acetonide in aqueous solution: influence of the cyclic ketal function. J Pharm Pharmacol 1983;35:175-177.

76. Beers M, Berkow R. The Merck manual of diagnosis and therapy. New Jersey: Merck Research Laboratories; 1999.

77. Lacy C, Armstrong L, Goldman M, Lance L. Drug information handbook. Tenth ed. Hudson: Lexi-Comp, Inc.; 2002.

78. Stanczak J, Blankenbaker D, De Smet A, Fine J. Efficacy of epidural injections of Kenalog and Celestone in the treatment of lower back pain. AJR Am J Roentgenol 2003;181(5):1255-1258.

79. Ciulla TA, Chriswell MH, Danis RP, Fronheiser M, Yuan P, TA C, et al. Choroidal neovascular membrane inhibition in a laser treated rat model with intraocular sustained release triamcinolone acetonide microimplants. Br J Ophthalmol 2003;87:1032-1037.

80. Spaide RF, Sorenson J, Maranan L. Photodynamic therapy with verteporfin combined with intravitreal injection of triamcinolone acetonide for choroidal neovascularization. Ophthalmology 2005;112(2):301-304.

81. Spaide RF, Sorenson J, Maranan L. Combined photodynamic therapy with verteporfin and intravitreal triamcinolone acetonide for choroidal neovascularization. Ophthalmology 2003;110(8):1517-1525.

82. Jonas JB, Kreissig I, Degenring R. Intravitreal triamcinolone acetonide for treatment of intraocular proliferative, exudative, and neovascular diseases. Prog Retin Eye Res 2005;In Press, Corrected Proof.

83. Larsson J, Zhu M, Sutter F, Gillies MC. Relation between reduction of foveal thickness and visual acuity in diabetic macular edema treated with intravitreal triamcinolone. Am J Ophthalmol 2005;In Press, Corrected Proof.

84. Al-Haddad CE, Jurdi FA, Bashshur ZF. Intravitreal triamcinolone acetonide for the management of diabetic papillopathy. Am J Ophthalmol 2004;137(6):1151-1153.

85. Bakri SJ, Kaiser PK. Posterior subtenon triamcinolone acetonide for refractory diabetic macular edema. Am J Ophthalmol 2005;139(2):290-294.

86. Conway MD, Canakis C, Livir-Rallatos C, Peyman GA. Intravitreal triamcinolone acetonide for refractory chronic pseudophakic cystoid macular edema. J Cataract Refract Surg 2003;29(1):27-33.

87. Jonas JB. Intravitreal triamcinolone acetonide for treatment of sympathetic ophthalmia. Am J Ophthalmol 2004;137(2):367-368.

88. Paganelli F, Cardillo J, Melo L, Oliveira A, Skaf M, Costa R. A single intraoperative sub-tenon's capsule triamcinolone acetonide Injection for the treatment of post cataract surgery inflammation. American Academy of Ophthalmology 2004;111(11).

89. Park CH, Jaffe GJ, Fekrat S. Intravitreal triamcinolone acetonide in eyes with cystoid macular edema associated with central retinal vein occlusion. Am J Ophthalmol 2003;136(3):419-425.

90. Rechtman E, Allen VD, Danis RP, Pratt LM, Harris A, Speicher MA. Intravitreal triamcinolone for choroidal neovascularization in ocular histoplasmosis syndrome. Am J Ophthalmol 2003;136(4):739-741.

91. Obata R, Iriyama A, Inoue Y, Takahashi H, Tamaki Y, Yanagi Y. Triamcinolone acetonide suppresses early proangiogenic response in retinal pigment epithelial cells after photodynamic therapy in vitro. Br J Ophthalmol 2007;91(1):100-4.

92. Smithen LM, Ober MD, Maranan L, Spaide RF. Intravitreal triamcinolone acetonide and intraocular pressure. Am J Ophthalmol 2004;138(5):740-743.

93. Scott IU, Flynn J, Harry W., Rosenfeld PJ. Intravitreal triamcinolone acetonide for idiopathic cystoid macular edema. Am J Ophthalmol 2003;136(4):737-739.

94. Kramer M, Ehrlich R, Snir M, Friling R, Mukamel M, Weinberger D, et al. Intravitreal injections of triamcinolone acetonide for severe vitritis in patients with incomplete Behcet's disease. Am J Ophthalmol 2004;138(4):666-667.

95. Jonas JB, Degenring RF, Kreissig I, Akkoyun I, Kamppeter BA. Intraocular pressure elevation after intravitreal triamcinolone acetonide injection. Ophthalmology 2005;112(4):593-598.

96. Krause H-J, Schwarz A, Rohdewald P. Polylactic acid nanoparticles, a colloidal drug delivery system for lipophilic drugs. Int J Pharm 1985;27(2-3):145-155.

97. Yeung C, Chan K, Chan C, Pang C, Lam D. Cytotoxicity of triamcinolone on cultured human retinal pigment epithelial cells: comparison with dexamethasone and hydrocortisone. Jpn J Ophthalmol. 2004;48:236-242.

98. Yeung C, Chan K, Chiang S, Pang C, Lam D. The toxic and stress responses of cultured human retinal pigment epithelium (ARPE19) and human glial cells (SVG) in the presence of triamcinolone. Invest Ophthal Vis Science 2003;44(12):5293-5300.

99. Beletsi A, Panagi Z, Avgoustakis K. Biodistribution properties of nanoparticles based on mixtures of PLGA with PLGA-PEG diblock copolymers. Int J Pharm 2005;298:233-241.

100. Berton M, Allemann E, Stein CA, Gurny R. Highly loaded nanoparticulate carrier using an hydrophobic antisense oligonucleotide complex. Eur J Pharm Sci 1999;9(2):163-170.

101. Niwa T, Takeuchi H, Kunou N, Kawashima Y. Preparations of biodegradable nanospheres of water soluble and insoluble drugs with D,L-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method and the drug release behaviour. J Control Rel 1993;25:89-98.

102. Leo E, Brina B, Forni F, Vandelli MA. In vitro evaluation of PLA nanoparticles containing a lipophilic drug in water-soluble or insoluble form. Int J Pharm 2004;278(1):133-141.

103. Song CX, Labhasetwar V, Murphy H, Qu X, Humphrey WR, Shebuski RJ, et al. Formulation and characterization of biodegradable nanoparticles for intravascular local drug delivery. J Control Rel 1997;43(2-3):197-212.

104. Zambaux MF, Bonneaux F, Gref R. Influence of experimental parameters on the characteristics of poly (lactic acid) nanoparticles prepared by a double emulsion method. J Control Rel 1998;50(1-3):31-40.

105. Jeong Y, Shim Y, Choi C, Jang M, Shin G, Nah J. Surfactant-Free Nanoparticles of Poly(DL-Lactide-co-Glycolide) Prepared with Poly(L-Lactide)/Poly(Ethylene Glycol). Journal of Applied Polymer Science 2003;89:1116-1123.

106. Murakami H, Kawashima Y, Niwa T, Hino T, Takeuchi H, Kobayashi M. Influence of the degrees of hydrolyzation and polymerization of poly(vinylalcohol) on the preparation and properties of poly(-lactide-co-glycolide) nanoparticle. Int J Pharm 1997;149(1):43-49.

107. Borges O, Corderio-da-Silva A, Romeijn SG, Amidi M, de Sousa A, Borchard G, et al. Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination. J Control Rel 2006;114:348-358.

108. Zhang Z, Feng SS. The drug encapsulation efficiency, in vitro drug release, cellular uptake and cytotoxicity of paclitaxel-loaded poly(lactide)-tocopheryl polyethylene glycol succinate nanoparticles. Biomaterials 2006;27(21):4025-33.

109. Miglietta A, Cavalli R, Bocca C, Gabriel L, Gasco MR. Cellular uptake and cytotoxicity of solid lipid nanospheres (SLN) incorporating doxorubicin or paclitaxel. Int J Pharm 2000;210:61-67.

110. Shaikh S, Ho S, Engelmann LA, Klemann SW. Cell viability effects of triamcinolone acetonide and preservative vehicle formulations. Br J Ophthalmol 2006;90:233-236.

111. Szurman P, Kaczmarek R, Spitzer MS, Jaissle GB, Decker P, Grisanti S, et al. Differential toxic effect of dissolved triamcinolone acetonide and its crystalline deposits on cultured human retinal pigment epithelium (ARPE 19) cells. Exp Eye Res 2006;83:584-592.

112. Bejjani RA, BenEzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, et al. Nanoparticles for gene delivery to retinal pigment epithelial cells. Mol Vis 2005;11:124-32.

113. Kwon SS, Nam YS, Lee JS, Ku BS, Han SH, Lee JY, et al. Preparation and characterization of coenzyme Q10-loaded PMMA nanoparticles by a new emulsification process based on microfluidization. Colloids and Surfaces A: Physicochemical and Engineering Aspects 2002;210(1):95-104.

114. Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Del Rev 1997;28(1):5-24.

115. Gorner T, Gref R, Michenot D, Sommer F, Tran MN, Dellacherie E. Lidocaine-loaded biodegradable nanospheres. I. Optimization of the drug incorporation into the polymer matrix. J Control Rel 1999;57:259-268.

116. Bilati U, Alleman,E, Doelker,E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. Eur J Pharm Sci 2005;24:67-75.

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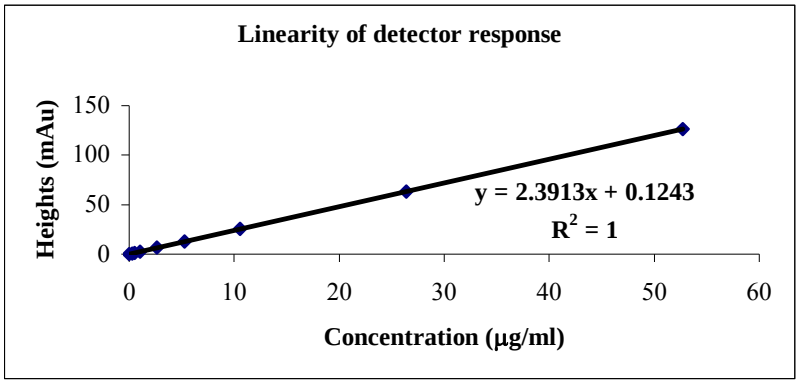
APPENDIX

Appendix 1: A sample of LOD/LOQ calculation



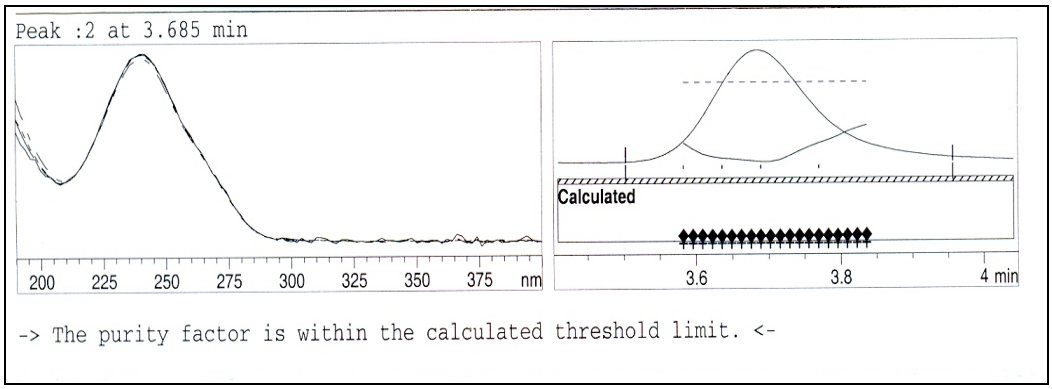
Noise :

Injection No.	Noise (6SD)
1	0.044
2	0.052
3	0.032
4	0.036
5	0.037
6	0.055
Average	**0.042**
SD	**0.009**

LOD
= (3 x Average of noise SD) : slope of std Conc vs height
= 0.053 (µg/ml)

LOQ
= (10 x Average of noise SD) : slope of std Conc vs height
= 0.18 (µg/ml)

Appendix 2: Purity factor of TA peak on HPLC assay



Appendix 3: Data of TA potency and degradation products

(a) TA stability in ACN: mobile phase 1:1 over 72 hours at three different temperatures

Time (h)	Potency (%)*			Degradation product (%)		
	4ºC	25ºC	37ºC	4ºC	25ºC	37ºC
0	100.00	100.00	100.00	0.00	0.00	0.00
3	100.00	99.19	99.44	0.00	0.81	0.56
6	99.62	98.68	98.54	0.38	1.32	1.46
9	100.00	98.84	97.98	0.00	1.16	2.02
24	100.00	98.77	96.33	0.00	1.23	3.67
72	95.88	95.48	97.31	4.12	4.52	2.69

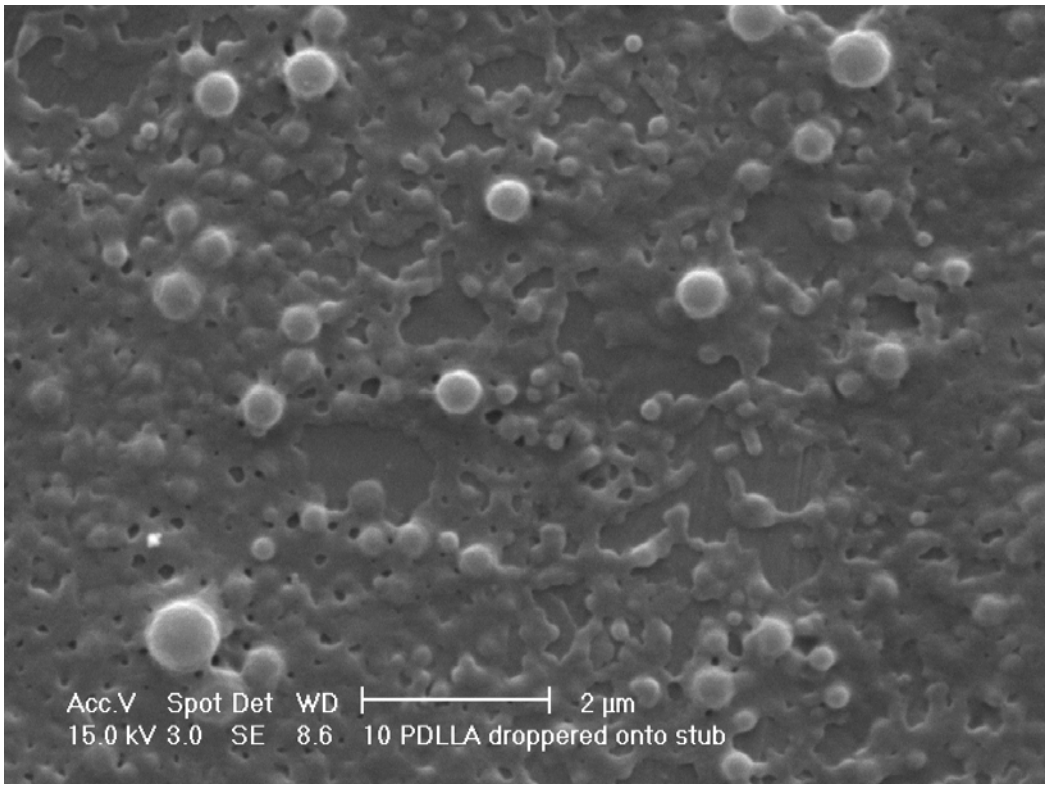
(b) TA stability in PBS pH 7.4 over 72 hours at three different temperatures

Time (h)	Potency (%)*			Degradation product (%)		
	4ºC	25ºC	37ºC	4ºC	25ºC	37ºC
0	100.00	100.00	100.00	0.00	0.00	0.00
3	97.90	97.37	95.68	2.10	2.63	4.32
6	97.33	96.46	94.53	2.67	3.54	5.47
9	99.00	98.00	94.29	1.00	2.00	5.71
24	97.21	92.80	84.49	2.79	7.20	15.51
72	95.73	88.44	60.23	4.27	11.56	39.77

(c) TA stability in PBS-NaN_3 over 72 hours at three different temperatures

Time (h)	Potency (%)*			Degradation product (%)		
	4ºC	25ºC	37ºC	4ºC	25ºC	37ºC
0	100.00	100.00	100.00	0.00	0.00	0.00
3	96.56	97.84	95.67	3.44	2.16	4.33
6	95.57	94.81	93.40	4.43	5.19	6.60
9	99.24	97.71	92.88	0.76	2.29	7.12
24	96.82	93.95	82.03	3.18	6.05	17.97
72	93.60	85.63	57.05	6.40	14.37	42.95

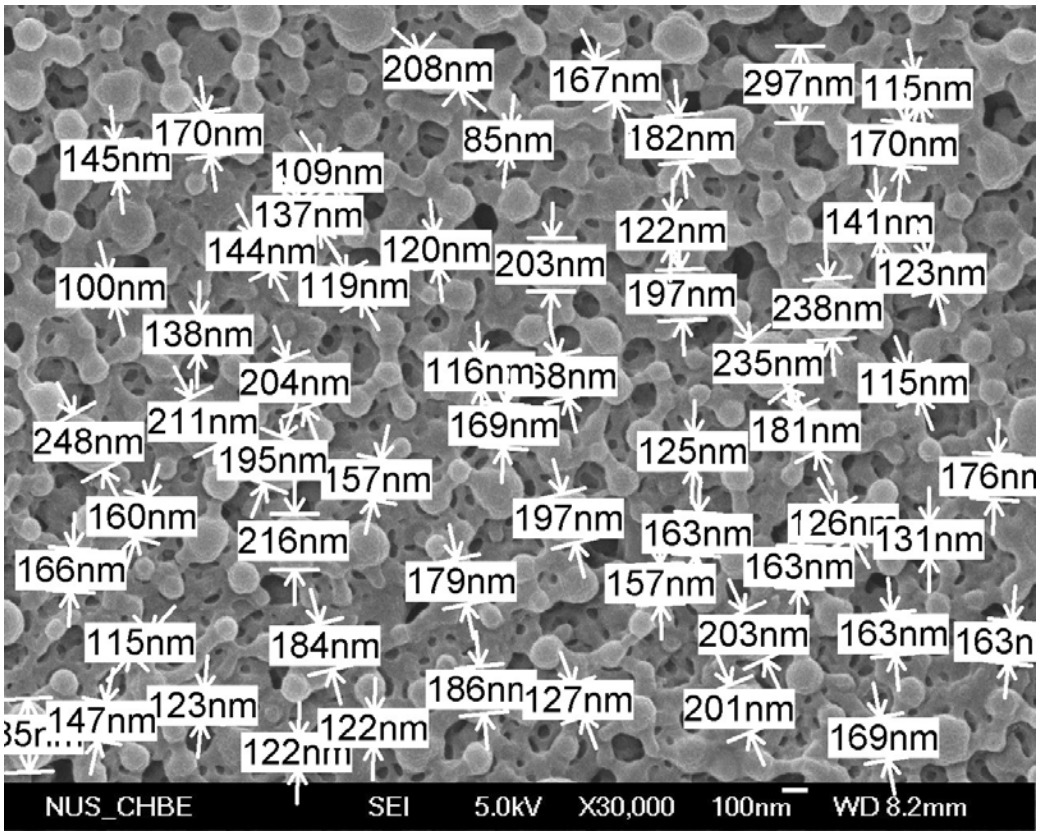
Appendix 4: SEM image of PDLLA NPs made by solvent diffusion method



Appendix 5: Turbidity data

Time (min)	Absorbance		
	TA	**PDLLA**	**mPEG PLGA**
0	0.003	3.786	3.767
2	0.015	3.889	3.806
5	0.389	3.603	3.697
10	0.370	3.852	3.842
15	0.309	3.994	3.999
20	0.268	3.555	3.690
25	0.379	3.903	3.920
30	0.350	3.727	3.998

Appendix 6: Particle measurement by FESEM on TA-MPEG PLGA



Appendix 7: Solubility data of TA in PBS-NaN_3 pH 7.4

Time (hour)	Solubility (μg/mL)
8	**6.58 ± 1.20**
24	**27.40 ± 0.40**
48	**28.06 ± 0.82**