Chapter 2

Literature review

2.1. Brain cancer and treatment

Glioma is grade IV neuroepithelial tumour, accounts for 80% of all primary CNS tumours [1]. Glioma is arising from glial cells that are normally providing nutrition and support to neurons in central nervous system (CNS). Brain cancer accounting for 2,38,000 new cases and 1,74,000 deaths estimated by World Health Organization (WHO) through international agency for research on cancer (IARC), GLOBOCAN 2008 [38]. In 2012 alone, 2,56,213 peoples were affected by brain and nervous system tumours worldwide accounting 1,39,608 men and 1,16,605 women [39]. Estimated deaths were calculated to be 1,89,382 peoples accounting 1,06,376 men and 83,006 women worldwide. Every day about 700 people are diagnosed with a maligant brain tumour. Glioma is metastatic in nature, diffusely penetrating throughout brain and extends far beyond the original tumour mass. Unfortunately, every last tumour cell is neither removed surgically nor killed by current treatment strategies. Glioma is associated with poor prognosis, frequent recurrence and extremely high lethality. Median survival of glioma patients after surgical removal was found to be only 3 months [3]. Radiotherapy and chemotherapy are found to prolong median survival of glioma patients only up to a year. Therefore, glioma treatment is a major challenge to clinicians and scientists.

2.2. Resveratrol

Resveratrol (RSV) is a natural molecule present in grapes, red wine, peanuts, berries and in several materials of normal human diet. At first, RSV was isolated from the roots of *Veratrum grandiflorum O. Loes* (white hellebore) in 1940 [5]. Latter, it was also isolated from the roots of *Polygonum cuspidatum* (Japanese knotweed) in 1963 [5, 6]. RSV exists in *cis* and *trans* forms. Among them, *trans* form of RSV (3,4,5-trihydroxystilbene) was proved to be biologically active (Figure 2.1). RSV is proved to be active in preventing myocardial infarction, cardioprotection, reducing platelet aggregation, vasodilation, prevents stroke and brain damage, minimize inflammation, reduction of cholesterol and triglycerides deposition in liver, prolongation of lifespan and cancer prevention [7]. RSV is also proved to inhibit several type of cancer cells such as head & neck cancer, breast, lung, thyroid, liver, colon, gastric, pancreatic, prostate, ovarian and muscle cancers. Recently, RSV is also proved for its anticancer potential against glioma [8-10].
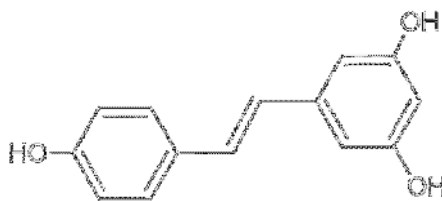

Figure 2.1. Molecular structure of *trans* resveratrol (3,5,4'-trihydroxystilbene)

2.3. Physico chemical properties of RSV

Appearance	:	Off-white colour crystalline powder
Molecular formula	:	$C_{14}H_{12}O_3$
Molecular weight	:	228.247 g/mol
Solubility (in water)	:	3 mg/100 mL
Log P value	:	3.06

2.4. Therapeutic potential of RSV

2.4.1. RSV against myocardial infarction and cardioprotection

RSV is proved for the protection of rat heart against ischaemia. Administration of RSV at 1 mg/kg for 15 days was proved to be sufficient to improve coronary blood flow in isolated hearts [40]. RSV administration with drinking water at 14 mg/kg for 16 days significantly increase cardiac QR1 (DT-diaphorase) and catalase levels in guinea pigs [41]. RSV perfusion at 10 µM concentration for 10 to 15 min before ischaemic induction results in reduction of malondialdehyde, improved aortic flow and reduction of infarct size [42, 43]. This effect of RSV against ischaemia is due to its antioxidant activity [44]. In cardiac homogenates and isolated atria, RSV prevented formation of reactive oxygen species induced by menadione. RSV is proved to increase the nitric oxide via increasing nitric oxide synthase expression and decreasing nitric oxide inactivation by free radicals.

These results suggest that RSV might protect against ischeamic damage during myocardial infarction.

'French Paradox' is a term to explain the low risk of cardiovascular disease due to consumption of red wine by French even with high fat diet [45-47]. Regular low consumption of alcoholic beverage is beneficial to cardiovascular health. Several studies indicate that red wine cause significant addition of benefits such as vasorelaxation [48, 49], reduction of platelet aggregation [50, 51], reduce atherosclerosis [52-54] and suppress lipid peroxidation [53]. These cardioprotective effects of red wine prompted to study about RSV.

2.4.2. RSV against platelet aggregation

Excessive aggregation of platelets causes thrombus formation and subsequent blockages of blood vessels that result in transient ischaemia, myocardial infarction or stroke. RSV prevents platelet aggregation both *in vitro* and *in vivo* [55, 56]. Prostacyclin is an antiplatelet aggregator synthesized by COX2 in vascular endothelial cells. In contrast, thromboxane A2 (TxA2) is a potent inducer of platelet aggregation. TxA2 is synthesized by COX1 in platelets. RSV is proved for selective inhibition of COX1 which causes decrease in TxA2 and thereby prevents platelet aggregation [57, 58]. Systemic administration of RSV is also proved to thwart platelet aggregation in rabbits induced by hypercholesterolaemic diet [59].

2.4.3. RSV against stroke and brain damage

Several studies reported the protective effect of RSV against brain damage due to cerebral ischaemia. Intraperitoneal (*i.p.*) injections of RSV for 21 days in rats showed smaller infarct volume after middle cerebral artery occlusion and lesser motor impairment [60]. In Mongolian gerbils, *i.p.* injection of RSV during and after transient global cerebral ischaemia decreased delayed neuronal cell death and glial cell activation in the hippocampus [61]. RSV administration at 100 and 1000 ng/kg in rats after middle cerebral artery occlusion significantly reduced ischaemic volume and brain water content [62].

2.4.4. RSV against oxidative stress and inflammation

RSV is reported to reduce oxidative stress and inflammation. Oxidative stress is due to reactive oxygen species (ROS) that are generated from electron transport chain of mitochondria and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. ROS damage biological macromolecules and thereby activate inflammatory mediators. Inflammation further increases oxidative stress and contribute for the progression of several diseases such as cancer, diabetes mellitus, atherosclerosis and pulmonary diseases [63-65]. The oxidative and ROS induced diseases can be prevented, treated or cured through resveratrol. Antioxidant effect of RSV may inhibit the oxidation of low density lipoprotein and thereby decreases the endothelial damage in cardiovascular diseases [66]. Administration of 100 mg of RSV and 75 mg of grape skin polyphenols with high fat and carbohydrate meal to 10 healthy humans significantly increased messenger RNA (mRNA) expression of NADPH and glutathione S-transferase

pi 1 genes. These observations strongly suggest anti-oxidant effect of RSV [67]. RSV administration also showed suppression of postprandial rise in cluster of differentiation 14 (CD14), IL-1 b mRNA, toll like receptor 4 (TLR4) protein in mononuclear cells and plasma endotoxin. These results clearly suggest that RSV reduces oxidative stress and inflammation. Therefore, RSV has potential to reduce risk of atherosclerosis and diabetes via these mechanisms.

2.4.5. RSV against obesity and diabetes

RSV is proved to improve insulin sensitivity and lowers body weight in diet induced obesity in rodent models. Animals treated with high RSV doses resulted in weight loss, ability to run farther and tolerate cold longer than the control animals [68]. RSV administration at 200 mg/kg/day for 1 year increased basal metabolic rate in nonhuman primate Microcebus murinus. These results suggest that RSV might increase energy consumption and promote weight loss. The mechanism of insulin sensitizing effect of RSV includes SIRT1-dependent suppression of protein-tyrosine phosphatase 1B, antiinflammatory effects and prevention of lipid deposition in muscle and liver [69-71]. Sirtris Pharmaceuticals introduced RSV formulation in 2008 and proved for higher glucose tolerance in type II diabetics in Phase 1 clinical trial [72]. RSV administration in patients aged 60-80 with impaired glucose tolerance for 4 weeks supports the insulin sensitizing effect. Fasting glucose level was unchanged without an increase in insulin production, which indicates the improved insulin sensitivity [72]. RSV supplementation in type II diabetic men at 5 mg/kg for 4 week improved insulin sensitivity, lowered blood glucose levels and delayed glucose peak in comparison to placebo [73].

2.4.6. RSV in vasodilation

In addition to vasorelaxant effect via inhibition of TxA2 synthesis, RSV is also proved for relaxation effect in isolated arteries and rat aortic rings [74, 75]. The mechanism of vasorelaxant activity of RSV was attributed to the stimulation of Ca^{2+} activated K^+ channels and enhancement of nitric oxide signaling in the endothelium. Nitric oxide was attributed to inhibit vascular NADH/NADPH oxidase activity which results in reduction of basal superoxide production. RSV showed expression of endothelial and inducible nitric oxide synthase (eNOS and iNOS) in experimental animals. These studies clearly indicate the vasorelaxant effect of RSV.

2.4.7. RSV against cholesterol and triglycerides deposition

RSV is shown to inhibit the deposition of cholesterol and triglycerides in the rat liver. RSV was also reduced the rate of hepatic triglyceride synthesis [76]. RSV administration is reported for reduction of total cholesterol concentration, serum LDL and very low-density lipoprotein concentrations in hypercholesterolaemic rats [57, 77]. RSV has been shown to decrease the formation of atherosclerotic plaques and restore flow-mediated dilation in rabbits fed with high-cholesterol diet [57]. RSV derivative resveratrol β-glucoside (piceid) present in red wine is one of the effective regulator of serum lipid concentrations [76].

2.4.8. RSV against cancer prevention and glioma

Chemopreventive effect of RSV was first identified in skin tumour in 1997 [78]. RSV was found to reduce the skin tumour of mouse up to 98%, which triggered research on

this molecule. RSV administration at 200 µg/kg/day in rats with colon cancer suggest that RSV concentration in dietary sources (red wine) could exert anticancer effect in some cases [79]. In another study, RSV at 40 mg/kg increased the survival of the mice with subcutaneous neuroblastoma up to 70%. Despite several studies were reported for potential chemotherapeutic effect of RSV, few studies at low concentration did not show anti cancer effects [80]. RSV administration at 1 - 5 mg/kg/day failed to prevent the growth and metastasis of breast cancer in mice. RSV at this low concentration showed potential cytotoxic effect in *in vitro* cell line studies. Several clinical trials are also being carried out to explore the chemopreventive effect in humans. RSV was proved to be significantly reduce the self-renewal and tumour-initiating capacity of glioma stem cells [7]. Nanog is one of the transcription factors for maintaining the phenotype and pluripotentency of embryonic stem cells. p53 regulates Nanog transcriptional activity by decreasing Nanog mRNA expression. RSV was proved to enhance phosphorylation and activation of p53 and there by downregulates Nanog mRNA that results in suppression of self-renewal and tumour-initiating capacity of glioma stem cells [7]. Several molecular mechanisms of RSV such as induction of G0/G1 cell growth arrest by suppression of cyclin D1 expression, p53-dependent apoptosis by essential binding to plasma membrane integrin αVb3, inhibition of angiogenisis by decreasing VEGF expression and suppression of tumour invasion by inhibition of matrix metalloproteinases (MMPs), cell growth inhibition via caspase-3 mRNA and caspase-3 activation were also reported against glioma [7]. RSV was showed to inhibit autophagy in glioma cells and thereby enhances therapeutic efficacy of other anticancer drugs such as temozolomide against

malignant glioma both *in vitro* and *in vivo* [81]. Moreover, RSV was proved to enhance radiosensitivity and thereby inhibit proliferation of human glioma cells [7].

2.5. Problem with RSV for therapeutic applications

The number of publications on RSV is triggered after proved for its chemopreventive effects. Though RSV possess strong efficacy against glioma, its therapeutic applications are greatly restricted because of its poor biological half life, rapid metabolism and elimination. Plasma half life ($t_{1/2}$) of RSV was found to be only 15 minutes after oral administration [14]. Intravenous administration (*i.v.*) also resulted in short $t_{1/2}$ of about 33 minutes [17]. Short half life, rapid metabolism and elimination necessitate higher dose and frequent administration for acquire the therapeutic potential of RSV. Many attempts have been made such as polymeric nanoparticles, solid lipid nanoparticles, polymeric lipid-core nanocapsules and β-cyclodextrin complexes and nanosponges to improve the half life and decreasing intensive metabolism [15]. Polymeric micelles and transferrin modified poly ethyleneglycol-poly lactic acid nanoparticle formulations also attempted to improve the therapeutic efficacy and brain targeting potential of RSV [82, 83].

2.6. Nanoformulations of RSV

Several nanoformulations were attempted for bioavailability enhancement, improving chemical stability and targeting of RSV to specific tissue target. RSV loaded glyceryl behenate solid lipid nanoparticles (SLN) were prepared by solvent evaporation technique for passive brain targeting. The SLN formulation having 248.30±3.80 nm showed approximately 5 times higher brain accumulation in comparison to pristine RSV after

intraperitoneal (*i.p.*) administration (Resveratrol formulations SLN1). In another study, RSV loaded SLN comprised Precirol ATO 5, palmitic acid, Gelucire 50/13 and tween 80 were prepared and surface coated with mucoadhesive N-trimethyl chitosan graft palmitic acid co-polymer. The mucoadhesive SLN showed 3.8 times higher bioavailability than that of RSV suspension after oral administration (SLN2). SLN formulation of RSV made up of glyceryl behenate, hydrogenated soybean lecithin and poloxamer 188 showed rapid penetration through the cell membrane of keratinocyte without any significant changes in cell morphology, metabolic activity and cell cycle [84].

RSV incorporated in mPEG-PLA nanoparticles showed significantly higher cytotoxicity lower production reactive oxygen species (ROS) in C6 glioma cells [85]. Poly(ε-caprolactone) nanoparticles containing RSV showed significantly higher cytotoxicity in murine melanoma cells, decrease the tumor volume, increase necrotic area and inflammatory infiltrate of melanoma in mice than that of RSV suspension [86]. Oral administration of RSV loaded PLGA nanoparticles showed 10.6 times higher area under the curve (AUC) and 2.78 times lower liver accumulation in comparison to pristine drug [87]. RSV loaded in nanoparticles comprised of poly(epsilon-caprolactone) and poly(D,L-lactic-co-glycolic acid)-poly(ethylene glycol) copolymer showed significantly higher cytotoxicity in prostate carcinoma DU-145, PC-3 and LNCaP cells [88]. RSV loaded chitosan nanoparticles surface modified either by biotin or by both biotin and avidin showed significantly higher accumulation in liver and higher cytotoxicity against HepG2 (hepatic carcinoma) cells [89]. Polymer-lipid hybrid nanoparticles (HNPs) of RSV showed significantly higher brain accumulation in rats after intraperitoneal (*i.p.*) and oral administrations. RSV loaded HNPs showed higher cytotoxicity against C6

glioma cell line and decrease in tumour size and reduced the incidence of some malignant tumour-associated characteristics, such as intratumoral hemorrhaging, intratumoral edema and pseudopalisading in C6 glioma cells implanted tumour model [90, 91].

RSV loaded proliposomal formulations using distearoyl phosphatidyl choline and cholesterol were prepared for oral bioavailability enhancement. AUC and C_{max} of RSV proliposomal formulations were found to be two fold higher than that of pristine RSV [92]. RSV transfersomes with different surfactants such as, polysorbate 80, sodium cholate and sodium deossicholate and RSV ethosomes (ethanol-containing vesicles) with soy phosphatidylcholine and cholesterol were prepared for the treatment of skin cancer. The vesicular formulations showed significantly higher cytotoxicity, lower production of reactive oxygen species (ROS) and lipid peroxidation, following incubation of H_2O_2-stimulated human keratinocytes (HaCaT) [93]. Co-encapsulation of RSV with curcumin in liposomes showed synergistic improvement of bioavailability and anti-tumour effect against prostate cancer after oral administration in prostate-specific PTEN-knockout mice [94]. Intravenous (*i.v.*) administration of liposomes at 5 mg.kg^{-1} body weight of RSV in nude Balb/c female mice with subcutaneous head and neck squamous cell carcinoma showed approximately 70% reduction in tumour volume [95].

RSV loaded O/W nanoemulsions showed higher penetration through Caco-2 cell monolayers. Pea nut oil and soy lecithin based nanoemulsions of RSV showed significantly higher chemical stability and antioxidant effect in comparison to pristine RSV [96, 97]. Halloysite is natural alumino silicate clay with hollow tubular structure. RSV loaded halloysite nanotubes showed controlled release and higher cytotoxicity

against MCF-7 breast cancer cell lines [98]. Colloidal mesoporous silica nanoparticles showed significantly higher cytotoxicity in HT-29 and LS147T colon cancer cell line in comparison to pure RSV via the PARP and cIAP1 pathways [99]. RSV loaded gelatin nanoparticles prepared using coacervation method showed significantly higher cytotoxicity in non-small cell lung carcinoma (NCI-H460) cells via increased Bax, p53, p21, caspase-3 protein levels, and decreased Bcl-2 and NF-kB proteins expression. RSV-gelatin nanoparticles also showed higher *in vivo* anticancer effect than free RSV in Swiss albino mice [100]. RSV loaded β-cyclodextrin nanosponges showed higher cytotoxicity in HCPC-I cells than that of pristine RSV. The RSV nanosponges also showed higher *ex vivo* accumulation of resveratrol in rabbit buccal mucosa and good permeation in pig skin which suggest the potential development of oral and topical delivery systems [101].

2.7. TPGS

D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) is a semi synthetic surfactant synthesized from esterification of natural vitamin E succinate with polyethylene glycol 1000. TPGS is a water soluble derivative useful in reversing or preventing vitamin E deficiency [102]. It is an amphiphilic molecule as shown in Figure 2.2. The hydrophilic lipophilic balance (HLB) value of TPGS is found to be 13, which can emulsify wide range of water - oil immiscible systems. TPGS has been reported for wide variety of pharmaceutical applications such as bioavailability enhancer, emulsifier, stabilizer and P-glycoprotein (P-gp) inhibitor. It is also useful material of prodrugs, fabrication of PLA-TPGS nanoparticles and TPGS based nanomedicines such as polymeric nanoparticles, liposomes and micelles. Nanoparticles prepared using TPGS takes the advantage of

polyethylene glycol (PEG) for its long half-life in circulation and vitamin E for its high cellular uptake owing to permeability [102-104]. This combined advantage is not available with nanoparticles prepared using PLA, PLA-PEG, PLGA and PLGA-PEG except PLGA-TPGS. However, very few works on nanoparticles were reported using PLGA-TPGS as matrix material in cancer diagnosis and therapy [105-107].
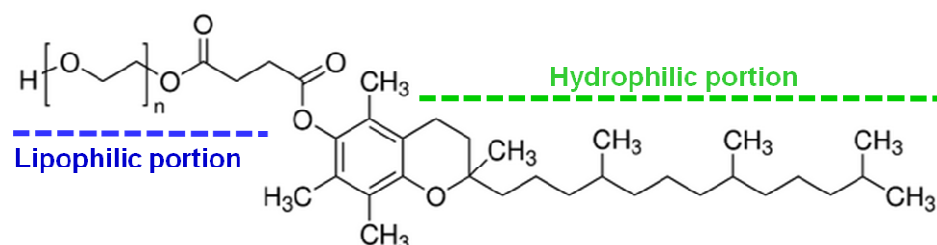

Figure 2.2. Molecular structure of TPGS showing hydrophilic and lipophilic portion.

2.8. TPGS based formulations in cancer diagnosis and therapy

2.8.1. TPGS emulsified PLGA nanoparticles

Firstly, TPGS reported for enhancing bioavailability of vitamin E [108]. Further, TPGS was used by Mu and Feng as emulsifier to fabricate paclitaxel-loaded PLGA nanospheres by solvent evaporation/extraction technique [109]. The comparison of TPGS with traditional chemical emulsifier poly vinyl alcohol (PVA) revealed a significant improvement in encapsulation efficiency with TPGS without any significant changes in surface morphology. XPS investigation demonstrated that there was residual surfactant (PVA) molecules remained on the surface of the nanoparticles whereas TPGS was washed out completely. TPGS has 67 times higher emulsification efficiency than PVA,

with a HLB value of ~13. TPGS can be useful to achieve up to 100% encapsulation efficiency and also enhance cellular uptake of nanoparticles thereby promote apoptosis [19, 110]. Enhancement in cytotoxicity, inhibition of P-gp mediated drug resistance and increase in oral bioavailability of anticancer drugs are some other supportive findings associated with co-administration of TPGS [22, 111-115]. It was also confirmed by Fourier Transform Infra-Red Photoacoustic Spectroscopy (FTIR-PAS) investigation of the nanospheres. The *in vitro* release studies indicated that the release profile of paclitaxel strongly depends on the emulsifier type employed in the fabrication. The authors reported that TPGS could be an ideal and effective emulsifier for the preparation of nanoparticles. TPGS was used as matrix material with PLGA nanoparticles [19, 116, 117]. TPGS was also used in 2:1, 1:1, 1:2 ratios as matrix material with PLGA and 0.015%, 0.03% and 0.06% as emulsifier/stabilizer. The effect of concentration of TPGS was studied in terms of particle size and entrapment efficiency. It was observed that the particle size was decreased from 895.4 to 686.1 nm as the TPGS concentration was increased from 0.015% to 0.06%. Encapsulation efficiency of nanoparticles was found to be 53.2% with 0.03% of TPGS (used as emulsifier). When TPGS was used as matrix material, encapsulation efficiency was reached up to ~100%. TPGS could also be used effectively as matrix material to enhance the entrapment efficiency [116].

TPGS emulsified paclitaxel PLGA nanoparticles was applied as an effective tool to overcome the side effects associated with the conventional paclitaxel injection. One of the major limitations associated with paclitaxel is its low aqueous solubility due to its extremely hydrophobic nature. Therefore, only injection is available as *i.v.* formulation in an adjuvant 50:50% v/v mixture of cremophor EL (polyethoxylated castor oil derivative)

and dehydrated ethanol. Before administration, it should be diluted 5–20 folds in normal saline or dextrose solution (5%). The adjuvant is found to have severe side effects including hypersensitivity reactions, neurotoxicity, nephrotoxicity and cardiotoxicity. Nanoparticles of biodegradable polymers could provide an ideal solution for the alternative formulation for conventional injection using cremophor EL, which can also provide sustained, controlled and targeted delivery of the drug. Application of TPGS in the development of PLGA nanoparticles of paclitaxel could improve the encapsulation of drug as high as 100%. *In vitro* toxicity studies and *in vivo* pharmacokinetic studies revealed superior efficacy than Taxol[®] [118]. Area under the curve (AUC) of TPGS emulsified PLGA nanoparticle formulation of paclitaxel was found to be 3 times more than that of conventional injection formulation. In another *in vivo* anticancer activity on male severe combined immuno deficiency (SCID) mice by xenograft model revealed that the PLGA nanoparticles of paclitaxel formulated using TPGS could be 4 and 1.5 times more effective than Taxol[®] in suppressing tumour growth on 21st and 31st day, respectively [119].

TPGS was also used in nanoparticles capable of photodynamic therapy (PDT). The advantage of nanoparticles towards chemotherapy is its high internalization into cancer cells than normal drug. A disadvantage of the nanoparticulate chemotherapy is due its retention in intracellular vesicles inside the cancer cells. The nanoparticle structure encapsulating anticancer drug is entrapped in endocytic vesicles formed in intracellular region of cancer cells and is unable to release the drug, where the drug can accomplish their biological activity [120]. PDT is a photochemical process for producing localized tissue necrosis and improves the treatment efficacy. PDT involves the activation of a

photo sensitizer or photosensitizing drug in the target tissue with specific wavelength light. The process of activation of photosensitizer or photosensitizing drug induces the formation of reactive oxygen species (singlet oxygen) [120]. Singlet oxygen is highly reactive and has a very short lifetime and short range of action (10-20 nm). This will rupture the endosomes and lysosomes and release the endocytosed content of nanoparticles into the cytosol. This is also termed as photochemical internalization. To address the multidrug resistance (MDR) the photodynamic internalization was coupled with P-gp inhibition by TPGS and proved for its enhanced photodynamic therapy. A biodegradable polymer (PLA) was combined with a photosentizer [meso-tetra-(p-hydroxy methylphenyl) porphyrin (m-THMPP)] to form 4-armed porphyrin-PLA and a nanoparticles formulation encapsulating doxorubicin (DOX) was prepared using TPGS as emulsifier [120]. A synergistic action was observed in the combination of photosensitizer and P-gp inhibitor with a chemotherapeutic drug in DOX resistant breast cancer cell line (MCF-7). The utilization of TPGS decreased the P-gp activity, increased the intracellular accumulation of DOX and thereby increased the therapeutic efficacy. Both TPGS and irradiation of the photoreactive nanoparticles caused DOX to move from the cytoplasm to the nucleus and causes increased accumulation than free drug [121].

2.8.2. Drug conjugated TPGS prodrug in cancer therapy

Polymer-drug conjugation is one of the major strategies to increase solubility, permeability and stability of drugs and thereby increasing their therapeutic efficacy. This also improves pharmacokinetics and pharmacodynamics, reduces their side effects as well as circumvents the MDR which is developed by over expression of multi drug

resistance proteins such as P-gp. Current medical research directs to co-administer P-gp inhibitors such as cyclosporine A or verapamil which may also suppress the body immune system and thus cause complications in therapy. Incorporation of these inhibitors in nanoparticles is also a major challenge in fabrication and stability aspects. Conjugation of drug with biodegradable polymer is an alternative strategy proved to inhibit MDR transporters of many chemotherapeutic drugs such as DOX, paclitaxel, camptothecin and platinum compounds [122, 123]. TPGS-DOX conjugation was prepared and cellular uptake; intracellular distribution and cytotoxicity were evaluated using MCF-7 breast cancer cells and C6 glioma cells as *in vitro* cell model [124]. The conjugate showed higher cellular uptake and broader distribution within the cells. The conjugate was found to be 84.1% more effective in MCF-7 cells after 72 h and 87.7% more effective in C6 cells after 48 h than the parent drug. The *in vivo* pharmacokinetics and biodistribution studies after *i.v.* administration at 5 mg DOX/kg body weight in rats showed 4.5 fold increase in the half-life and 24-fold increase in the AUC of TPGS–DOX conjugate in comparison to the free DOX. The drug level in heart, gastric and intestine was reduced markedly, which is an indication of reduced side effects [124].

2.8.3. TPGS coated liposomes in cancer diagnosis and therapy

TPGS is used to modify the surface characteristics of the liposomes and thus alter the bio-distribution. Liposomes were useful for controlled and sustained delivery of both hydrophilic drug and lipophilic drugs. Passive targeting by EPR effect and active targeting by ligand conjugation were also proved using nanoliposomes [125-127]. To overcome some of the drawbacks associated with conventional liposomes such as

opsonisation by immune systems and faster elimination from blood circulation, stealth liposomes i.e. PEG coated liposomes were formulated and proved for their stability and longevity in blood [128]. Longevity is due to PEG chain, which prevents the adsorption of plasma protein on the liposomes surface and identification of liposomes by immune system [129]. Recently, liposomes surface modified with TPGS was prepared by solvent injection method using docetaxel as model drug and possibility against brain tumour was compared by *in vitro* cell line studies with PEGylated liposomes, conventional nude liposomes (without TPGS coating) and marketed formulation of docetaxel (Taxotere[®]) (Figure 2.3). Promisingly, TPGS coated liposomes showed 7 times more efficacy than Taxotere[®]. TPGS liposomes showed the IC_{50} value of only 5.93±0.57 µg/ml whereas PEGylated liposomes, uncoated liposomes and Taxotere[®] showed 7.70±0.22, 31.04± 0.75 and 37.04 ±1.05 µg/ml, respectively, after 24h culture with C6 glioma cells. Cell uptake studies using coumarin-6 on C6 glioma cells were also showed better internalization of TPGS liposomes than other formulations. The better efficacy and higher cell uptake of TPGS liposomes was attributed to its passive transport by EPR effect and inhibition of P-gp mediated drug efflux by TPGS [20].
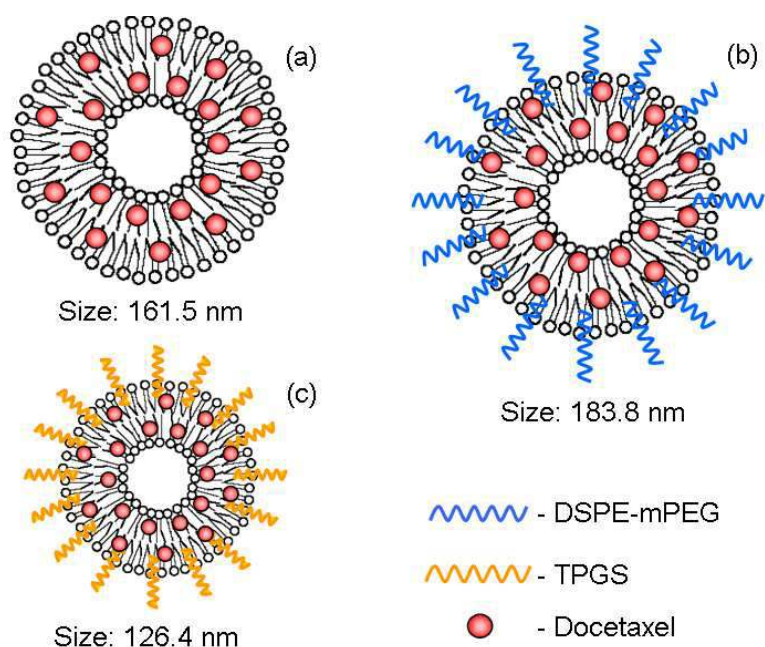
Figure 2.3. Schematic diagram of (a) conventional liposomes (b) PEG-coated liposomes and (c) TPGS-coated liposomes.

In another study, Muthu *et al*. prepared TPGS coated multifunctional liposomes containing docetaxel and QDs with and without targeting moieties (Figure 2.4). Folic acid was used as targeting probe to target folate receptor over expressing MCF-7 breast cancer cell lines. *In vitro* cell line studies were performed to assess cellular uptake and cytotoxicity of the drug and QDs loaded liposomes. The IC_{50} was found to be approximately 6 and 41 times lesser than that of Taxotere[®] for non-targeting and targeting liposomes, respectively, after 24 h culture with MCF-7 cells. Higher cellular uptake of targeting liposomes than the non-targeting was confirmed by CLSM images using MCF-7 cells after 2 h incubation [58].
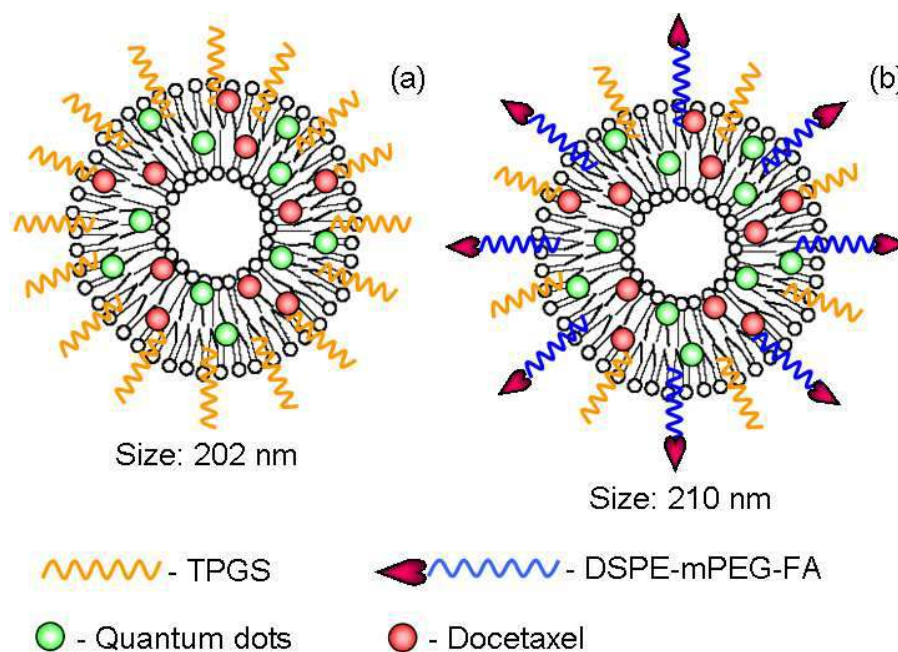
Figure 2.4. Schematic diagram of (a) multi-functional nontargeted liposomes (b) multi-functional folate receptor targeted liposomes.

A novel TPGS decorated emodin liposomes were formulated and compared with methoxy polyethyleneglycol 2000-derivatized distearoyl-phosphatidylethanolamine (mPEG 2000–DSPE) liposomes. TPGS decorated liposomes improved the cytotoxicity of emodin on leukemia cells and also prolonged the circulation time in blood. TPGS decoration of liposomes improved the AUC to 1.7 times longer than free emodin and 0.91 times larger than for mPEG 2000-DSPE liposomes [130].

2.8.4. TPGS in micelles for cancer diagnosis and therapy

Most of the potent anticancer drugs such as paclitaxel, docetaxel, tamoxifen are of low aqueous solubility and this being a major limitation to achieve good therapeutic efficacy. Various drug delivery systems such as nanoparticles, liposomes, dendrimers and micelles

were evaluated to solve this problem and also to improve sustained, controlled and targeted delivery of anticancer drugs. Among them, micelles can encapsulate these poorly aqueous soluble drugs in their hydrophobic core and can improve their bioavailability and therapeutic efficacy. Micelles showed passive targeting to tumour site through enhanced permeability and retention effect because of its small size ranging from 10 to 100 nm and presence of leaky vasculature at the tumour site [131]. Long circulation of drug encapsulated micelles can also be achieved by its hydrophilic surface which playing a key role to escape from recognition of RES. Because of small size range and EPR effect of micelles, they can provide high drug accumulation at the tumour site and thereby exert higher therapeutic efficacy and lower side effects in chemotherapy. Docetaxel loaded TPGS micelles ranged between 12 and 14 nm were prepared and proved for its improved anticancer efficacy in comparison to Taxotere[®] in C6 glioma brain cancer cells. IC_{50} value of micelle formulation of TPGS was found to be approximately 3 times lower than that of Taxotere[®] in C6 glioma cells. Mean plasma concentration of docetaxel after 2 h of *i.v.* administration was significantly higher for the TPGS micelles than Taxotere[®] [132].

TPGS is reported as solubilizer, absorption enhancer and a vehicle for lipid-based drug delivery formulations. Lipophilic portion of TPGS is relatively bulky which would improve the solubility of camptothecin [133]. Therefore, mixed micelles comprised of Pluronic and TPGS were prepared to acquire the advantages of TPGS for higher encapsulation and improved anticancer efficiency. Cytotoxicity of mixed micelles was evaluated in comparison to free drug and pluronic micelles on MCF-7 cell line. Mixed micelles showed significantly superior cytotoxicity in comparison to free drug and pluronic micelles [133].

In another study, TPGS micelles containing superparamagnetic iron oxide formulation were prepared and proved for improved thermal and magnetic properties, *in vitro* cellular uptake, lower cytotoxicity and better *in vivo* imaging effects in comparison to commercial Resovist[®] and Pluronic F127 micelles [134]. The prepared micelles were found to be highly monodisperse, water soluble, preferred size range and more importantly stable in 0.9% normal saline for a period of 12 days. Cellular uptake and cytotoxicity were investigated *in vitro* in MCF-7 breast cancer cell lines. T2 mapped images of xenograft grown on SCID mice showed that the TPGS micelle formulation of iron oxide had ~1.7 times and ~1.05 times T2 decrease at the tumour site compared to Resovist[®] and the F127 micelle formulation, respectively [134].

Recently, TPGS 2k using tocopheryl succinate and PEG 2000 (mPEG 2000) were prepared with folate receptor targeting (Figure 2.5). This attempt has been made based on the concept that increasing chain length prevents the recognition from reticulo endothelial system (RES). Promisingly, TPGS 2k showed much lower CMC value compared with traditional TPGS and formed physiologically stable micelles with TPGS 2k alone without addition of any other polymers or lipids. TPGS 2k showed approximately 10 times lower CMC value of 0.0219 mg/ml in comparison to traditional TPGS (0.2 mg/ml) [135]. The research group successfully formulated folate decorated micelles using DOX as model drug and proved for its improved targeting and cell internalization than its marketed formulation Taxotere[®] using MCF-7 breast cancer cells *in vitro*. The docetaxel-loaded TPGS 2k micelles with and without folate conjugation were of desired size and size distribution, high drug encapsulation efficiency and favourable drug release. The improved targeting efficacy was demonstrated based on IC_{50} value, which is the drug

concentration required for 50% cell viability. The IC_{50} of TPGS 2k micelle was found to be 99.5%, 80.4% decrease and 57.5% increase in comparison to Taxotere® in 24, 48, 72 h, respectively. Folic acid conjugated micelles showed 99.8%, 88.1% and 23.0% lower IC_{50} value in comparison to Taxotere® in 24, 48, 72 h, respectively,.
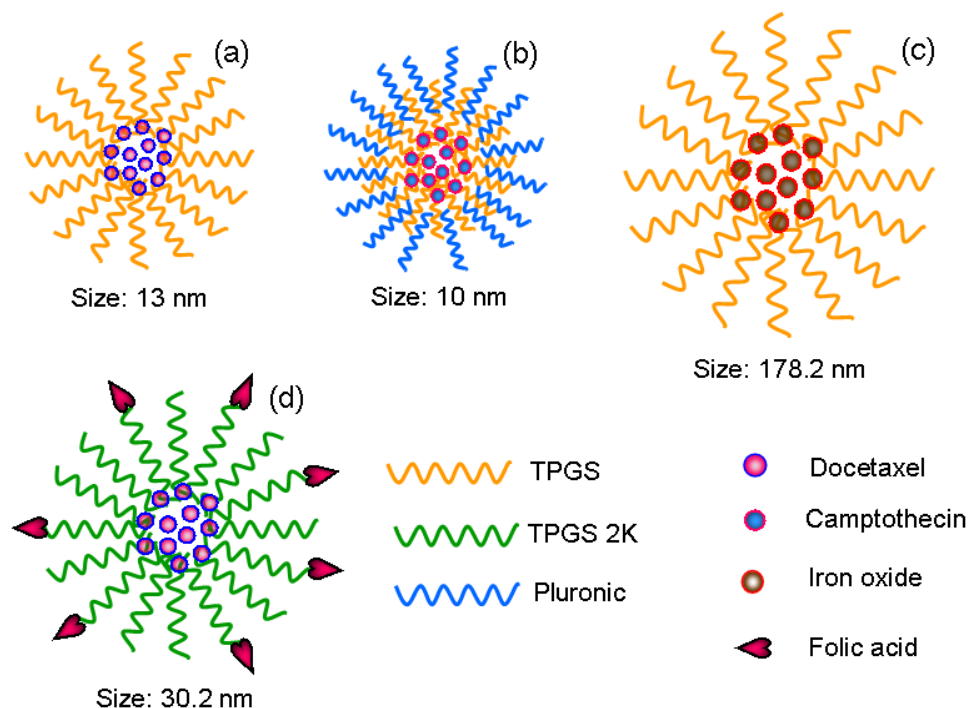

Figure 2.5. Schematic diagram of (a) TPGS micelles encapsulating docetaxel (b) TPGS and Pluronic mixed micelles encapsulating camptothecin (c) TPGS micelles encapsulating iron oxide (d) folate-targeted TPGS 2k micelles encapsulating docetaxel.

2.9. TPGS copolymer based nanomedicine in cancer diagnosis and therapy

The drawbacks of polymers such as PLA and PLGA in nanoparticles preparations are high hydrophobicity and slow degradation. Therefore, advantages of TPGS can be

utilized for triggering nanoparticles for faster drug release by improving the biodegradability of PLA/PLGA polymers. The faster degradation was achieved by synthesizing co-polymers such as PLA-TPGS and PLGA-TPGS. [136]. PLA-TPGS copolymers were synthesized by ring-opening bulk polymerization of lactide monomer with TPGS in the presence of stannous octonate as catalyst. It was reported that nanoparticles prepared with PLA-TPGS copolymer improved paclitaxel entrapment efficiency up to 91.5%. The drug release from PLA-TPGS nanoparticles was found to be 17% and 51% of the encapsulated drug in day 1 and after 31 days, respectively, whereas PLGA nanoparticles showed only 7% and 19% drug release, in the same time periods [136]. It was claimed that the faster drug release of PLA-TPGS nanoparticles might be caused by lower molecular weight and the higher hydrophilicity of copolymer in comparison to PLGA. Higher hydrophilicity of PLA-TPGS caused more swelling and faster degradation and thus promoted the drug release from the nanoparticles. The cellular uptake in HT-29 cells was improved from 26.8% (PLGA nanoparticles emulsified with PVA) to 53.1% (PLA–TPGS nanoparticles emulsified with TPGS) [137]. The higher uptake of PLA-TPGS might be due to inhibition of P-gp, present at the surface of cancer cell.

The novel PLA–TPGS nanoparticle formulation of paclitaxel also showed significant advantages in achieving higher cytotoxicity and smaller IC_{50} value over Taxol[®]. Pharmacokinetic studies revealed PLA-TPGS nanoparticle formulation is promising for sustainable chemotherapy. PLA-TPGS nanoparticle formulation achieved 27.4 fold longer half life and 1.6 fold higher area under the curve (AUC) in comparison to marketed Taxol[®] injection [138]. No portion of plasma concentration time curve was

located above the maximum tolerated dose (8,540 ng/ml) of paclitaxel, whereas, 39.9% of plasma concentration time curve of Taxol® injection was located above the maximum tolerated dose, which may cause severe side effects. In nanoparticles based chemotherapeutic research, one shot for 240 h therapy was achieved with PLA-TPGS in comparison to Taxol® injection (only 22 h) at the same 10 mg/kg of paclitaxel dose. *In vivo* anti-tumour efficacy of PLA-TPGS in xenograft tumour model was two times more effective than Taxol® injection. Though no formulation showed successful treatment of HT-29 tumours *in vivo* in the literature, PLA–TPGS nanoparticle formulation demonstrated its potential efficacy in controlling the tumour growth and 100% survival rate. Synthesis of PLA-TPGS self assembled nanoparticles prevented desorption of TPGS from nanoparticle surface and increased the stability of TPGS on the nanoparticles surface. PLA-TPGS self assembled paclitaxel nanoparticles increased the cellular uptake (nearly 2 fold) in comparison to PLGA nanoparticles prepared using TPGS as emulsifying agent. IC_{50} of the PLA–TPGS nanoparticle formulation of paclitaxel with HT-29 cells was found to be 40% lower than that of Taxol® [139].

Active targeting can be useful to achieve high tumour uptake of nanoparticles and thereby improve the therapeutic efficacy and reduce the side effects of chemotherapy. PLA-TPGS nanoparticles were applied effectively for specific tumour targeting using surface modification with targeting probes (Figure 2.6). Folate receptor is over expressed in the cell membrane of many tumours of brain, kidney, breast, ovarian and lung cancer cells. The expression of folate receptors in tumour cells was 100 to 300 times more than the normal cells [140-142]. Folic acid is an essential vitamin for the synthesis of neclotide bases, which playing a major role in proliferation of cancer cells. In targeted

therapy, PLA-TPGS and TPGS-COOH copolymer were used for increasing half life of nanoparticles in blood stream and to facilitate the folate conjugation on the surface of nanoparticles. Folate decorated nanoparticles exhibited superior targeted delivery to cancer cells and improved the therapeutic efficacy. Cellular uptake was 1.5 fold high in folate decorated nanoparticles than bare nanoparticles. Folate decorated nanoparticles showed intense green fluorescence of courmarin-6 around the nucleus in comparison to weak fluorescence of nontargeted nanoparticles [143].
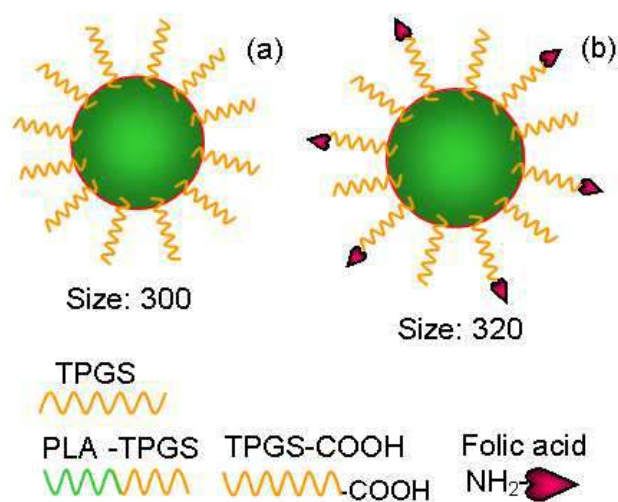

Figure 2.6. Schematic diagram of (a) Non-targeted PLA-TPGS nanoparticles using TPGS as emulsifier (b) targeted PLA-TPGS nanoparticles emulsified with folate targeting.

Transferrin (Tf) conjugated nanoparticles of PLA-TPGS containing docetaxel was also reported for targeted delivery across blood brain barrier (BBB) [144]. The BBB selectively allows the amount of chemotherapeutic agents into the brain and hence being a major obstacle in the treatment of brain tumour. Almost all the drugs including most of

anticancer molecules cannot penetrate the BBB. Drugs with high liphophilicity can usually penetrate through BBB in trace amounts [145, 146]. Conventional invasive strategies such as intra cerebral or inter ventricular delivery are more painful, unsafe, expensive and high possibility of infections [147]. Therefore, non-invasive strategies were developed without damaging brain tissues to overcome the restrictions of BBB. Among them, receptor mediated transport is an attractive strategy because of circumvention of multi-drug resistant caused by P-gp efflux pump, high specificity, low immunological response and the ability to deliver macromolecules such as protein and peptide drugs across BBB [148]. Tf receptor is expressed in luminal side of the capillaries serving blood to the brain and over expressed in many types of brain tumours. Tf has been extensively studied for its receptor mediated transport and shown to be a promising molecular probe for targeted drug delivery to the brain [149]. Surface modified nanoparticles with Tf should be 100-200 nm in size to deliver drug through EPR effect and sufficiently hydrophilic in order to escape from opsonisation. The efficiency of Tf conjugated PLA-TPGS nanoparticles were investigated in close comparison with bare PLA-TPGS nanoparticles formulation as well as with the marketed formulation of docetaxel (Taxotere®). IC_{50} data showed that the Tf-conjugated PLA-TPGS nanoparticles formulation of docetaxel could be 16.9% and 229% more efficient than the PLA-TPGS nanoparticles formulations and marketed clinical formulation (Taxotere®) after 24 h treatment, respectively. *Ex-vivo* bio-distribution studies demonstrated that the Tf-conjugated PLA-TPGS nanoparticles formulation could be able to deliver the therapeutic agents across BBB [144].

Herceptin[®] conjugated PLA-TPGS:TPGS-COOH copolymer blend was evaluated for its targetability to SK-BR-3 and MCF7 breast cancer cells [150]. Ligand conjugation can be done before (preconjugation strategy) or after (post conjugation strategy) formation of nanoparticles. Post-conjugation strategy was followed to fabricate Herceptin[®] conjugated PLA-TPGS:TPGS-COOH copolymer blend nanoparticles. In addition to simple post conjugation process, the size control was achieved by varying the ratio of PLA-TPGS: TPGS-COOH copolymer blend and the quantity of ligand were also controlled by varying feeding concentration of ligand in Herceptin[®] conjugation process. The positive correlation was obtained between the surface density of the ligand and the cellular internalization as well as the cytotoxicity of the nanoparticle formulations, which demonstrated that the strategy developed in that research was simple and feasible for precise control of targeting effects.

Diagnosis of cancer tumours at its earlier stage is still a major challenge for clinicians. Iron oxide (IOs) nanoparticles have been investigated as magnetic resonance imaging (MRI) contrast agent, which can be absorbed, digested and eliminated by human body [151]. PLA-TPGS copolymer was used as carrier to fabricate biodegradable nanoparticles containing IOs for medical imaging of cancer tumour. Biocompatibility study and cellular uptake in MCF-7 breast cancer cells and NIH-3T3 mouse normal fibroblast and *in vivo* tumour uptake studies were performed in close comparison with the commercial MRI imaging iron formulation Resovist[®] [118]. For MCF-7 breast cancer cells, the cellular uptake of iron oxide PLA-TPGS nanoparticles was found to be 20.4 fold higher than that of Resovist[®] at the equivalent iron concentration of 0.75 mM Fe/L. Cellular uptake of iron oxide formulated as PLA-TPGS nanoparticles was found to be only 6.5 fold higher

than the Resovist® formulation in NIH-3T3 mouse fibroblast cells. The noteworthy finding was more accumulation of iron from PLA-TPGS nanoparticles in MCF-7 cancerous cells than the normal cells of NIH-3T3 mouse fibroblast. MRI imaging of tumour site in xenograft tumour model confirmed the advantages of the PLA-TPGS nanoformulation versus Resovist®. MRI imaging was further carried out to investigate the biodistribution of both PLA-TPGS nanoformulation of iron oxide and Resovist®. It was found that the PLA-TPGS nanoformulation of IOs at the clinically approved dose of 0.8 mg Fe/kg could be cleared within 24 h in comparison with several weeks for Resovist®. The clearance of IOs PLA-TPGS nanoformulation was confirmed from the loss of signal intensity in liver on T2 weighed MRI image [118].

QDs are widely studied as luminescence probes for imaging cancer cells in recent years. It possess several advantages over conventional chemical fluorescent imaging agents such as tunable emission wavelength from visible to infrared by controlling its size and composition, high quantum yield of fluorescence, strong brightness and photostability. In spite of several advantages of QDs over bare flouorescent probes their applications in medical field were restricted due to its inevitable toxic effects. Many efforts have been made in this field over the past decade for the practical applicability of QDs in imaging. Coating of QDs with amphiphilic polymers and encapsulating with dendrimer like compounds were reported to reduce the cytotoxicity [152, 153]. Encapsulation of QDs in PLA-TPGS copolymer greatly improved biocompatibility stability and realised for controlled and sustained release of QDs from the polymeric matrix [154]. Another strategy was developed for targeted delivery of QDs, which can target selectively to the cancer cells by passive and active targeting modes. Size of the nanoparticles

encapsulating QDs was reduced to 100-200 nm to attain the targetability through EPR effect [155]. Active targeting can be achieved by either coating with polymers which are conjugated with targeting probes or by encapsulating QDs in biodegradable polymeric nanoparticles which are surface modified with targeting ligands. PLA-TPGS: TPGS-COOH copolymer blend nanoparticles surface modified with folic acid encapsulating QDs were prepared with various weight ratios. TPGS-COOH was conjugated with folic acid in this formulation, which will ultimately decide the amount of folate ligand conjugated onto the nanoparticles surface. Targetability of QDs loaded PLA-TPGS/TPGS-COOH nanoparticles was studied in both MCF-7 breast cancer cells which are over-expressing folate receptors and NIH 3T3 fibroblast cells which are expressing low quantity of folate receptors [156]. Confocal laser scanning microscopy (CLSM) studies demonstrated higher internalization of folate-decorated QDs-loaded PLA-TPGS/TPGS-COOH nanoparticles by MCF-7 breast cancer cells than the cellular uptake by NIH 3T3 fibroblast. Folate targeted nanoparticles showed lower cytotoxicity in both MCF-7 cells and NIH 3T3 cells. Additionally, cytotoxicity of QDs formulated in the PLA-TPGS/TPGS-COOH nanoparticles was lower for normal cells such as NIH 3T3 cells than that for MCF-7 breast cancer cells due to folate targeting effect. Therefore, copolymers of TPGS conjugated with targeting moieties will be a promising tool for targeted delivery of nanoparticles containing chemotherapeutic drugs as well as QDs. Multimodal imaging system by co-encapsulating superparamagnetic IOs for MRI and QDs fluorescence imaging in the nanoparticles of PLA-TPGS were fabricated to combine their advantages and to promote a sustained and controlled imaging with passive targeting to the cancer cells. This novel strategy not only reduced the toxicity of the

individual contrast agents but also improved their biocompatibility and cancer cell uptake. Co-encapsulation of MRI and fluorescent imaging probes shielded the contrast agents from detection by the human immune system and thus increasing their half-life in blood circulation and realized sustained and controlled delivery of imaging agents with passive targeting effects to the tumour site [157].

2.10. DSPE PEG 2000 and PEGylated nanoparticles

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE PEG 2000) is a potential molecule frequently reported for the preparation of stealth nanoparticles [34, 158]. DSPE PEG 2000 and allied derivatives coated nanoparticles were well proved for prolonged systemic circulation and bioavailability enhancement of many therapeutic molecules [159, 160]. DSPE PEG 2000 coated cationic liposome was prepared to deliver oxaliplatin to both tumor endothelial cells and tumor cells in a solid tumor. Multiple injections of PEGylated liposomes induced higher apoptotic activity in tumor tissue and resulted in superior intratumor distribution [161]. DSPE PEG 2000 coated liposomes containing Gadolinium (a magnetic resonance imaging detectable liposome) showed high relaxivity, stability and biocompatibility [162]. Paclitaxel liposomes coated with DSPE PEG 2000 showed sustained release and prolonged systemic circulation time in comparison to Tween 80 coated liposomes in rats [163].

Adsorption of blood proteins (opsonisation) causes recognition/engulfing of nanoparticles by phagocytes called phagocytosis. Therefore, attempts were made to prevent the

adsorption of blood proteins on the surface of the nanoparticles. Surface coating of nanoparticles with PEG (PEGylation) renders stealth nature and proved for avoiding phagocytosis. Surface modification of nanoparticles with PEG can be effectively achieved by synthesis of PLA-PEG copolymer. He *et al*. synthesized two types of triblock copolymers; ABA type (PLA-PEG-PLA) and the BAB type (PEG-PLA-PEG) [164]. These amphiphilic polymers formed nanomicelles in aqueous medium in the size range of 40-200 nm. The copolymers could be able to entrap 35% of paclitaxel by weight on the average. The nanomicelles prepared using BAB type of copolymer showed more controlled release than the ABA type nanomicelles; moreover, the actual release rates are influenced by the PLA chain lengths. Surface PEG contents influence the "stealth" characteristics of the nanomicelles. Compared with PLA particles, all nanomicellar particles of both BAB and ABA types showed four-fold reduction in monocyte cell uptake.

In another study, Dong and Feng synthesized methoxy poly(ethylene glycol)-poly(lactide) copolymer (mPEG-PLA) and fabricated nanoparticles by nanoprecipitation method for controlled release of paclitaxel [165]. The prepared nanoparticles were found to be spherical in shape with size less than 100 nm and X-ray Photon correlation Spectroscopy (XPS) analysis proved the presence of PEG layer on the nanoparticle surface. The prepared nanoparticles showed 85% drug release in first 7 days and 90% drug release in 14 days. The facilitated release was claimed due to its high surface to volume ratio of mPEG-PLA nanoparticles and presence of hydrophilic polymeric matrix which facilitates the penetration of water into the polymeric matrix. It was proved that the prepared nanoparticles are of core-shell structure comprised of PLA in core and

hydrophilic PEG in shell. This structure is believed to possess self stabilization of nanoparticles and expected to have long-circulation effects in blood stream. PLA-PEG nanoparticles surface modified with targeting ligands have shown better targeting efficiency than uncoated nanoparticles. Further, Hu *et al*. formulated coumarin-6 loaded PLA-PEG nanoparticles surface modified with lactoferrin for brain targeted drug delivery [166]. The uptake of surface modified nanoparticles was significantly higher than the unconjugated nanoparticles. Intravenous administration (*i.v.*) of lactoferrin conjugated nanoparticles showed 3 times more accumulation in mice brain than unconjugated nanoparticles and the surface modified nanoparticles was suggested to be a good carrier for drug delivery to the brain [166]. In another study, PLA–PEG–PLA tri block copolymer synthesized using ring opening polymerization reaction was also reported for nanoparticles encapsulating anticancer drugs such as 5-fluorouracil and paclitaxel. The novel triblock polymeric nanoparticles were reported for *in vitro* release for 12 days in controlled manner for both the drugs [167].

Poly lactic acid/ poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) copolymer (PLA/PEG-PPG-PEG) nanoparticles containing camptothecin was prepared and proved for its improved anticancer efficacy. The AUC (0–∞) value (134±19 ng.h/ml) of camptothecin solution was slightly greater than that of nanoparticles (127±20 ng.h/ml). The MRT (0–∞) were much larger for nanoparticles (25.1 ± 3.2 h) than for camptothecin solution (0.623 ± 0.157 h). Moreover, the nanoparticle formulation showed significantly higher tumour suppression than camptothecin solution on *i.v* administration to the mice bearing sarcoma 180 (S-180) solid tumour [168].

Phagocytosis is facilitated by the adsorption of plasma proteins (opsonins) to the particle surface. The effect of PEG grafting density in nanoparticles on plasma protein binding and *in vitro* macrophage uptake was investigated using rhodamine B loaded PLA-PEG nanoparticles prepared from 1:1 (wt/wt) blend of PLA and PLA-PEG copolymer of varying PEG grafting density (1, 7 and 20% mol/mol of lactic acid monomer is used to formulate PEG 1%-g-PLA, PEG 7%-g-PLA, PEG 20%-g-PLA nanoparticles) [169]. Dynamic light scattering technique was used to investigate the adsorption of plasma protein on to the surface of the nanoparticles which can be done by monitoring the size distribution of nanoparticles before and after incubation for a specific period of time with bovine serum albumin (BSA) or Foetal bovine serum (FBS). After 24 h of incubation of simple PLA, PEG 1%-g-PLA, PEG 7%-g-PLA, PEG 20%-g-PLA nanoparticles, the particle size was measured. Among them, PEG 7%-g-PLA, PEG 20%-g-PLA nanoparticles did not showed any significant increase in particle size. While PLA and PEG1%-g-PLA nanoparticles showed clear aggregation evidenced by the larger size and broader polydispersity index obtained after incubation. The results strongly support that either PEG7%-g-PLA or PEG20%-g-PLA nanoparticles did not adsorb higher quantities of plasma proteins compared to either PLA or PEG1%-g-PLA nanoparticles. Macrophage cellular uptake of the above formulated nanoparticles was measured in macrophage cell lines. The cell monolayers in 24-well flat bottom plates were incubated with nanoparticle formulations encapsulating rhodamine B for 24 h at 37 °C. The cell monolayers were washed, lysed with 0.2% Triton-X 100 in 0.2 N sodium hydroxide solution and measured for fluorescent intensity using fluorescence microscopy. Cells exposed to PLA and PEG 1%-g-PLA nanoparticles showed higher fluorescence intensity corresponding to higher

cellular uptake compared to cells exposed to either rhodamine B loaded PEG7%-g-PLA or PEG20%-g-PLA nanoparticles. This finding indicated that nanoparticles made up of PEG-g-PLA showed lesser internalization by macrophage cells than that of PLA nanoparticles. Moreover, it was demonstrated that the minimum concentration required to achieve sufficient masking by PEG on the surface of the nanoparticles, the grafting density should be higher than 1% to obtain lower internalization by macrophage cells [169].

Polymeric micelles were found to be a good carrier for anticancer drugs because of their good biocompatibility, entrapment of hydrophobic drugs, high tumour accumulation via EPR effect because of its nano size range. For example, Xiao *et al*. formulated PLA-PEG micelles and found that the micelles were internalized in to the cells via lipid raft/caveolae-mediated pathway [170]. Moreover, PLA-PEG micelles reversed the multi drug resistance via inhibition of P-glycoprotein function and alteration of cell membrane microenvironment. The research group also suggested the dyanamin and caveolin dependant but clathrin independent endocytosis for the cellular uptake of PLA-PEG nanoparticles [171]. A pH responsive copolymer based on PLA, poly (ethylene glycol) methyl ether-b-(poly lactic acid-co-poly (b-amino esters) (MPEG-b-(PLA-co-PAE) block copolymer was synthesized and proved for its improved drug delivery to the cancer cells [172]. The novel copolymer was self-assembled into core/shell micelles in aqueous solution at low concentrations (i.e., low critical micellar concentration [CMC]). The pH-responsive copolymer was insoluble in blood pH 7.4, but soluble at pH lower than 6.5 because of protonation of amino group. The cytotoxicity studies showed that the copolymer had low toxicity whereas the doxorubicin (DOX) loaded micelles remained

high toxicity for Hep G2 cells. The mPEG-PLA-b-Polyarginine (R15) triblock copolymeric micelles were also reported for improved siRNA delivery to nude mice xenografted MCF-7 tumours [173].