Chapter 5

Discussion

5.1. SYNTHESIS AND CHARACTERIZATION OF NANOPARTICLES

Green tea polyphenols (GTP) are extensively investigated for their possible use in the field of medicine due to their major health benefits. As described in the earlier chapters (chapter 1 and 2), application of GTP as a therapeutic agent has been limited due to its poor bioavailability, stability and biotransformation (Zhu et al., 1997; Lu et al., 2003; Chow et al., 2005). This thesis we investigated the possibility of using GTP as a therapeutic agent by encapsulating the same with biodegradable nanoparticles, thereby increasing its bioavailability and stability. Nanoparticles were synthesised from biodegradable materials including polymers (combination of PLA and PEG), liposomes and proteins (casein and albumin) and GTP was loaded into these nanoparticles. In all the cases, particles ≤ 250 nm were observed through AFM and HR SEM (Figure 4.1, 4.2, 4.6, 4.7, 4.11, 4.12, 4.18 and 4.19). All the particles were observed to be spherical in shape. Mean hydrodynamic diameter of > 250 nm was observed through DLS (Table 4.2, 4.6, 4.9 and 4.13). The larger size (> 250 nm) of the particles could be due to aggregation of the particles within the dispersion medium. Size of the particles plays a significant role in some of the applications of nanoparticles such as delivery of loaded drug to the target site for cancer treatment. It has been reported that the pore size of a human colon adenocarcinoma is about 400 nm. Thus, for the treatment of human colon adenocarcinoma, a nanocarrier having a diameter of < 400 nm would be significant (Yuan et al., 1995). In our study, particles ≤ 250 nm was observed (through AFM and HR SEM) for all types of particles synthesised. Surface charge (ζ potential) of the particles were analysed through zeta potential analysis. All the particles were observed to be negatively charged. For liposomal and casein nanoparticles, the surface charge was in the range of -22 to -29 mV (Table 4.6 and 4.9), while in case of PLA-PEG and albumin nanoparticles, it was in the range of -63.7 to -74.9 mV and -66.3 to -84.9 mV respectively (Table 4.2 and 4.13). Therefore, the PLA-PEG and albumin nanoparticles are much stable than liposomal and casein nanoparticles.
FTIR analysis for GTP, albumin and GTP loaded albumin nanoparticles were carried out to observe the binding of GTP to albumin nanoparticles. A GTP specific distinct peak (2935.6 cm⁻¹) was observed in GTP loaded albumin nanoparticles, indicating the incorporation of GTP to albumin nanoparticles (Figure 4.16). XRD results were also corroborated with FTIR results showing the binding of GTP to albumin nanoparticles. As reported by Wei et al (2014), three peaks (2Θ = 21.3, 31.7 and 46.6°) were observed for albumin nanoparticles. Similar results were obtained in our study observing the three similar peaks (2Θ =21.3, 31.7 and 46.6°) for albumin nanoparticles. For GTP loaded nanoparticles, an extra peak was observed (2Θ=45.54°), which might be due to the presence of GTP within the nanoparticles (Figure 4.17).

To further confirm the incorporation of GTP to albumin nanoparticles, spectroscopic studies such as UV-vis spectroscopy, circular dichroism and fluorescence spectroscopy was carried out. An increase in the absorption intensity in UV-vis spectroscopy was observed for GTP loaded albumin nanoparticles compared to free albumin and unloaded albumin nanoparticles (Figure 4.18). This change in absorbance might be attributed to the conformational changes in albumin on loading of GTP (Pant et al., 2014). Circular dichroism results showed differences in the spectra for albumin, albumin nanoparticles and GTP loaded albumin nanoparticles. On loading of GTP to albumin nanoparticles, an increase in the negative bands at 210 and 220 nm was observed (Figure 4.19). Results showed that due to interaction of GTP with albumin nanoparticles, the α helical content of the protein (albumin) decreases, resulting in loose conformations of albumin with extended polypeptide chain. This conformational transition might be due to the exposure of hydrophobic areas to hydrophilic environment (Pant et al., 2014). Among the several techniques available to study protein drug interaction, the most convenient method is to study the fluorescence quenching. In our study, fluorescence spectroscopy was carried out to confirm the incorporation of GTP to albumin nanoparticles. The fluorescence of albumin is mostly due to tryptophan moiety which has a strong emission band at 350 nm when excited with a wavelength of 278 nm. A distinct peak was observed at 350 nm for albumin and on loading with GTP, the peak was blue shifted ~50 nm with a decrease in the fluorescence intensity (Figure 4.20). The decrease in the intensity might be related to its quenching. This quenching could be due to energy transfer,
molecular rearrangement or ground state complex formation (Mariam et al., 2011). This might also be due to the interaction between GTP and albumin. When albumin is exposed to a wavelength of 278 nm, tryptophan and tyrosin residues of the protein molecule get excited, reflecting upon the protein tertiary structure (Pant et al., 2014). Therefore, this change in fluorescence intensity before and after loading of GTP to albumin nanoparticles might point to the change in the protein conformation of albumin, particularly its tertiary structure (Pant et al., 2014). Therefore, increase in the UV-vis absorption, changes in the CD spectra and decrease in the fluorescence intensity in the presence of GTP, indicate that GTP has been incorporated to albumin nanoparticles.

Interaction between the constituents of GTP and albumin were studied through molecular docking. Most of the interactions between albumin and polyphenols were observed with the amino acids lysine and leucine. (Table 4.14) The docking studies of the major constituents of GTP with albumin revealed binding of GTP with albumin without the requirement of other compounds is in accordance with the studies reported by Weber et al (2000).

5.2. LOADING EFFICIENCY

Drug loading efficiency (LE) was determined for each of the particles. Among the four different particles studied, albumin nanoparticles showed maximum LE (96.4%) when GTP loading concentration was 10 mg/ml, (Table 4.12). For all the particles studied, it was observed that as the loading concentration of GTP was increased, there was an increase in the LE till a particular concentration and a decrease in the LE was observed on further increasing the concentration of GTP. Liposomal and casein nanoparticles showed LE of 77.5 and 76.9% for GTP concentration of 5 mg/ml. In PLA-PEG nanoparticles, for maximum loading of GTP, the ratio between polymer and drug should be about 8:1. Increase or decrease of PLA-PEG ratio affected loading of GTP. For different ratios of PLA-PEG, the LE also varied with maximum being 74.19% for PLA-PEG ratio of 3:1 at a GTP loading concentration of 12 mg/ml, which was further decreased on increasing the concentration of GTP. Therefore, it could be said that once the maximum LE is reached, the binding sites for GTP within the nanoparticles are exhausted and are not available for further binding of GTP.
5.3. IN VITRO RELEASE PROFILE OF GTP LOADED NANOPARTICLES

In vitro release studies were carried out for all the four particles for different loading concentrations of GTP, at different pH and also at different temperatures. In case of PLA-PEG nanoparticles, release studies were carried out for three different ratios (1:3, 1:1 and 3:1) of PLA-PEG. The rate of GTP release was faster with increased quantity of PEG (PLA-PEG ratio of 1:3) (Figure 4.3.1). As quantity of PEG decreased, slow and sustained release was observed. As PEG is more hydrophilic in nature, it affected diffusion rate of the drug, and thus the drug release (Chen et al., 2007). It was observed that as the PEG content increased, due to the hydrophilic PEG segment, the amount of GTP released also increased (Yang et al., 2009). It has been shown that the drug release is diffusion controlled, as the drug can travel through the pores formed during sphere hardening. When feed ratio of PLA increased, the shell of nanoparticles become thicker, which slows down the diffusion of drug (Das et al., 2001; Ruan et al., 2003). In our study, the amount of GTP released throughout the release period was affected by the composition of the nanoparticles, with the release being increased when PEG content of nanoparticles increased (Figure 4.3.1C). This observation suggests that in case of polymeric nanoparticles with a high PLA content, the release of GTP decreases. Release of GTP was monitored at different pH conditions of the release medium. In case of oral drug delivery systems, as the drug passes through the gastro intestinal tract (GIT), most of the drug is released due to the acidic pH of the GIT, thereby decreasing the bioavailability of the drug in target tissue. The major concern for the researchers is to restrict the drug release in gastric environment i.e., acidic pH to increase the bioavailability of the drug in target tissue. In our study, release of GTP was carried out in acidic conditions (pH 3) and it was observed that maximum amount of GTP was retained within the nanoparticles in acidic condition for all the nanoparticles studied with albumin nanoparticles showing the best result. In case of PLA-PEG nanoparticles, less amount of GTP was released in acidic pH (pH 3) compared to pH 7.4 and 9 (Figure 4.3.2). Similar results were obtained in case of liposomal (Figure 4.7B) and casein nanoparticles (Figure 4.11B). In case of albumin nanoparticles, the GTP release was observed to be less in acidic conditions compared to pH 7.4 and 9 (Figure 4.22B). Maximum amount of GTP was retained within the nanoparticles under acidic conditions, which may lead to increased
bioavailability of GTP at the target site. Cumulative percentage of GTP release was observed to increase as the pH shifted from acidic to basic. This indicates that as the pH increases the interaction between the drug carrier and the ligand (GTP) become weak, thereby increases the release of the GTP.

Release studies were further carried out by altering the temperature. As the temperature increased, an increase in the cumulative percentage of GTP release was observed for all the nanoparticles studied which indicates the possible role of temperature in the release of GTP. Maximum GTP was released at a temperature of 45°C compared to other temperatures (room temperature and 37°C). For PLA-PEG and liposomal nanoparticles, higher temperature probably influenced the structure of PLA-PEG and liposomal nanoparticles or affected the bonding between the particles and GTP thereby releasing higher percentage of GTP. In case of casein and albumin nanoparticles, it has been reported that protein based particles have no fixed structures and due to the lack of a rigid three dimensional structure, any changes in temperature may alter its structure (Lohcharoenkal et al., 2014). Thus the observed increase in the cumulative percentage of GTP release from nanoparticles at 45°C would be due to the alterations in the particles’ structure at higher temperature.

5.4. EFFECT OF ALCOHOL ON RELEASE OF GTP FROM GTP LOADED CASEIN AND ALBUMIN NANOPARTICLES

In case of protein nanoparticles i.e., casein and albumin nanoparticles, the influence of different concentrations of alcohol on dose dumping of GTP was also studied. The impact of concomitant intake of alcohol on drug release has become an increasing concern (Roth et al., 2009) because of its potential dose dumping. Consumption of alcohol along with oral dosage forms have been shown to undergo premature disintegration and unloading the complete dose. This phenomenon known as dose dumping can lead to increased plasma concentrations and drastic side effects of drugs with narrow therapeutic index. Dose dumping is regarded as harmful to the patient if the released dose is close to the toxic dose. Numerous works have been reported to determine the potential effects of alcohol induced dose dumping. Walden et al., (2007) showed the influence of alcohol on the release of hydromorphone, dihydrocodeine, morphine, oxycodone, codeine, and tramadol from different oral sustained release formulations. It was observed that hydromorphone alone in the
presence of 40% alcohol resulted in dose dumping, where 100% of the drug was released within an hour, while the other formulations showed consistent release over a longer period of time. Most frequently consumed alcoholic drinks (beer, wine and spirits) have alcoholic concentrations of about 5 - 40% so accordingly we have chosen the concentrations of alcohol for the study. The results of this study showed no dose dumping in any of the cases up to an alcoholic concentration of 40% in both casein and albumin nanoparticles. A sustained release was observed throughout the release period (up to 48 h), but as the percentage of alcohol in the release medium increased an increase in the cumulative percentage of GTP release was observed compared to that of PBS (Figure 4.23). Dose dumping was not observed in any of the cases, a maximum of 97% of the GTP was released in 48 h when alcohol was used at a concentration of 40% for casein nanoparticles, while that of albumin nanoparticles a maximum of 82% cumulative release was observed, which was higher than that of the control (PBS alone showed 72% cumulative release in 48 h). Thus, as the concentration of alcohol in the release medium increased, the cumulative percentage of GTP release also increased and it could be said that alcohol consumption affects the release of GTP from nanoparticles without dose dumping.

One of the methods for the quantification of alcohol induced dose dumping is by carrying out the $f_2$ similarity test. Similarity factor ($f_2$) is the logarithmic reciprocal square root transformation of the sum of the squared error (Morre and Flanner., 1996). The $f_2$ values usually range between 0 and 100. The dissolution profiles are considered similar if the $f_2$ value exceeds 50 and that it is alcohol resistant, while that below 50 indicated that the formulation is not alcohol resistant (Shah et al., 1998). In our study with GTP loaded albumin nanoparticles, all the dissolution profiles showed $f_2$ values > 50 and were therefore considered as alcohol resistant. Relative change in the amount of dissolution of alcoholic media with that of non alcoholic medium was evaluated, which provides a means of quantifying the dose dumping effect. Positive values indicate that alcohol increased the dissolution, while negative values indicate that alcohol decreases dissolution. In our study, PBS with 10% alcohol showed a negative value, while the other release media were positive, indicating increased dissolution, corroborating the results of the similarity test.

To develop an alcohol resistant modified release formulation, certain physical factors of the formulation components are to be considered. Some of the key factors like
swellability, media uptake capacity and wettability have been studied. Swelling property is one of the factors which influences alcohol induced dose dumping. In our study, GTP loaded hydrophilic polymer (albumin nanoparticle) was used to prepare modified release dosage form. It is known that a hydrophilic polymer (GTP loaded albumin nanoparticle) consists of a compressed mixture of polymer and drug. As the mixture comes in contact with water, a gel layer is formed due to polymer transition from glassy state to rubber state (Maderuelo et al., 2011). An alcohol resistant formulation was obtained when diffusion of alcohol to the polymer is prevented which in turn results in the thermodynamic equilibrium of polymer and the medium, resulting in the faster formation of gel layer. In our study with GTP loaded albumin nanoparticles, swelling percentage was observed to increase on increasing the percentage of alcohol in the release medium. Therefore, presence of alcohol in the release media hindered the gel formation, thereby not reaching the thermodynamic equilibrium fast enough. Media uptake capacity in our study with GTP loaded albumin nanoparticles could be correlated with the solubility and swelling property of the GTP loaded albumin nanoparticles and was much higher compared to that of non-alcoholic medium (Table 4.18).

Wettability of GTP loaded albumin nanoparticles was studied through drop shape method. Generally wettability is determined by measuring the contact angle of the liquid and the surface. A contact angle < 90° indicates wettability, while that > 90° indicate poor wettability. A contact angle close to 0° corresponds to excellent wettability (Jedinger et al., 2015). In our study with GTP loaded albumin nanoparticles, wettability was examined physically by the shape of the drop. It was observed that all the samples showed wettability property as the contact angle was < 90° (Table 4.18).

5.5. MATHEMATICAL MODELLING AND RELEASE KINETICS
The in vitro GTP release data was fitted into various kinetic models to determine the GTP release mechanism. It was observed that all the GTP release profiles showed good correlation with Zero order kinetics, where the concentration of GTP release and time are independent. Literatures state that the ideal model of drug release for nanoparticulate dosage forms or sustained release formulations is the Zero order kinetics (Costa and Lobo., 2001). Therefore, the nanoparticles studied in this thesis
(PLA-PEG, liposomal, casein and albumin nanoparticles) could be used as an ideal carrier for the delivery of GTP, achieving a prolonged or a sustained release system. According to Korsmeyer-Peppas, a pure Fickian release has a release exponent ‘n’ value limiting to 0.5, 0.45 and 0.43 for release from slabs, cylinders and spheres (Martinez et al., 2011). If value of ‘n’ is ≤ 0.43 it is considered as pure Fickian release where the release is through diffusion and that ranging from 0.43-0.89 is considered as anomalous transport which involves a coupling of diffusion and erosion mechanism and that > 0.89 is referred to as super case II transport where the transport is characterized by polymer relaxation due to polymer erosion when enzymatic degradation occurs (Arifin et al., 2006). In case of PLA-PEG nanoparticles of ratio 1:3, anomalous mode of drug release was dominated with the release mechanism being a coupling of diffusion and erosion mechanisms, while in case of PLA-PEG ratios of 1:1 and 3:1, Fickian mode of drug release was predominant, where the drug release is diffusion controlled (Table 4.3, 4.4, 4.5). In case of liposomal, casein and albumin nanoparticles, predominance was observed in anomalous mode of drug transport, which involves a coupling of erosion and diffusion mechanisms (Table 4.8, 4.10 and 4.15). In case of albumin nanoparticles, as the pH was shifted from acidic (pH 3) to basic (pH 9), the drug release mechanism was also shifted from anomalous transport to super case II transport which was in accordance with Serra et al (2006, Table 4.15). It could be said that the occurrence of super case II transport was due to enzymatic degradation of the polymer at a higher pH.

5.6. ANTIOXIDANT PROPERTY OF RELEASED GTP

In in vitro antioxidant studies, all the released samples showed effective radical scavenging activity. Since GTP is known to be a good antioxidant source, there was a direct correlation between the concentration of GTP released and its antioxidant activity. As the concentration of GTP release was maximum at about 4-5 h in all the nanoparticles studied (PLA-PEG, liposomal, casein and albumin nanoparticles), proportionally there was an increase in the radical scavenging activity (Figure 4.4, 4.8, 4.13 and 4.25). Since all the released samples showed the scavenging activity, GTP loaded nanoparticles used in this study would serve as a potent antioxidant source.
Among all the four nanoparticles studied (PLA-PEG, liposomal, casein and albumin nanoparticles), albumin nanoparticles were observed to exhibit better results with respect to its loading efficiency, minimum release of GTP in acidic condition and fitting most of its release data into Zero order kinetics (an ideal model for release of drugs from nanoparticulate systems and prolonged release formulations). Due to these considerations, the remaining work in this thesis was carried out using albumin nanoparticles.

5.7. RELEASE OF GTP IN SIMULATED BIOLOGICAL FLUIDS

*In vitro* release of GTP from albumin nanoparticles was carried out in simulated conditions of saliva (SSF, pH 6.5), gastric fluid (SGF, pH 1.2), intestinal fluid (SIF, pH 6.8) and colon fluid (SCF, pH 7) in fasted and fed states following the guidelines of US Pharmacopeia (Marques 2004; Marques et al., 2011). SSF, SGF, SIF and SCF represent the simulated conditions of saliva, stomach, intestine and colon respectively. In case of oral drug delivery systems, as the drug passes through the stomach, most of the active component of the drug is metabolized due to the acidic pH of the stomach (pH 1.2), thereby decreasing the bioavailability of the drug in the target site. To simulate the complete process of digestion or the drug metabolism in the gastrointestinal tract (GIT), starting from mouth to the stomach and to the colon, the release of GTP was monitored at different time at various regions of the GIT. During the process of digestion, maximum time food remains in saliva is 1 min, in stomach is ~2 h, while it takes ~ 4-6 h to pass through the intestine and it remains in colon for ~12-24 h based on the food taken. Similar time periods were considered in our study for GTP loaded albumin nanoparticles. The results of our study showed that a maximum of 36% and 51% of GTP was released (cumulative percentage of release) after 24 h on successive exposure to SSF, SGF, SIF and SCF in fasted and fed state respectively (Figure 4.24). Compared to fed state, release of GTP from albumin nanoparticles in fasted state resulted in more amounts of GTP to be entrapped within the nanoparticles. Therefore, through our study, it is evident that even after complete process of digestion, maximum amount of GTP is entrapped within the nanoparticles and could be delivered to the target site. The best fit model for the release of GTP in simulated conditions of saliva, gastric, intestinal and colon fluids was observed to be the Zero
order kinetics, which was in accordance to the in vitro release at different pH conditions, with the mechanism of release being an anomalous mode (Table 4.19).

5.8. IN VITRO CYTOTOXICITY STUDIES
To access the cytotoxicity of free GTP and GTP loaded albumin nanoparticles, MTT assay was carried out on normal (non cancerous) cells (3T3 cell lines) and two different cancer cell lines, MCF 7 (breast cancer cells) and PC 3 (prostate cancer cells). Albumin based nanocarriers have been widely studied as a delivery system for the delivery of several drugs, especially for those used in the treatment of cancer. Targeting of these drugs to tumour specific site involves an interaction between the drug molecule and the cell surface receptors. This approach enhances the therapeutic index and also develops the enhanced permeability and retention effects (Luo and Prestwich., 1999). The in vitro cytotoxicity of GTP loaded albumin nanoparticles were carried out and were compared with that of free GTP. No significant toxicity was observed in 3T3 cells for both free GTP and GTP loaded albumin nanoparticles in any of the tested concentrations (Figure 4.27). But the observed increase in the cytotoxicity of GTP loaded nanoparticles at 50 µg/ml for MCF 7 and all concentrations for PC3 could be the result of better uptake of the drug by cancer cells (Figure 4.26). Literatures show that the normal size of a tumour vessels ranged between 200 nm to 2 µm (Hashizume et al., 2000). In our study, the mean particle diameter of GTP loaded albumin nanoparticles were < 200 nm. Therefore, it could be said that GTP loaded albumin nanoparticles would have a better uptake by tumour cells in vivo.

5.9. BIOAVAILABILITY AND PHARMACOKINETICS IN IN VIVO (RABBIT MODEL)
Bioavailability of free GTP and GTP loaded albumin nanoparticles were studied in rabbit model. In this study, it was observed that GTP loaded albumin nanoparticles significantly increased the oral bioavailability of GTP compared to that of free GTP (Figure 4.28). As discussed earlier (Chapter 1), one of the limitations of using GTP as a therapeutic agent is its poor bioavailability. On loading of GTP in albumin nanoparticles, the bioavailability is increased. One of the possible reasons for this increase is that on oral administration of free GTP, most of the GTP get metabolized.
before it reaches the systemic circulation vis-a-vis the target site due to harsh pH conditions of the stomach juices, and/or the reticulo endothelial system (Elzohhby et al., 2012). On loading of GTP into albumin nanoparticles, GTP has been protected from degradation in pH conditions of the stomach juices and due to the small size of the GTP loaded nanoparticles, they bypass the reticulo endothelial system and more amount of drug reaches the systemic circulation and is available to the target site (Zhang et al., 2008). In the previous study carried out on in vitro release of GTP from albumin nanoparticles in simulated conditions (Section 4.4.5), very less amounts of GTP was released in simulated conditions of saliva, stomach, intestine and colon and maximum GTP was retained within the nanoparticles. These results corroborated with the in vivo results where the plasma GTP concentration was significantly higher compared to the plasma concentration of free GTP (Figure 4.28). On studying the pharmacokinetics of GTP and GTP loaded albumin nanoparticles, it was observed that C_{max} and T_{max} of GTP loaded albumin nanoparticles was higher than that of free GTP. Increase in half life of GTP in albumin nanoparticles, indicates the presence of GTP within the systemic circulation for a longer duration, which would serve as a base for designing the dosage regime and also the dosage intervals. Increase in AUC_{0-\infty} and MRT values and decrease in k_{a} values of GTP loaded albumin nanoparticles indicates the sustained release effects of GTP from albumin nanoparticles (Table 4.21) (Attia et al., 2007). A sustained release effect was also observed in in vitro release studies and also the release kinetics (which followed Zero order kinetics, an ideal model for release of drugs for sustained release formulations) (Table 4.20). The possible mechanism could be that GTP is carried through the gastro intestinal tract where it is protected from harsh pH conditions and the liver where GTP is protected from the metabolism in liver, thereby presenting GTP to the target site. Therefore albumin nanoparticles act as a carrier of GTP and also govern its slow release, achieving a sustained release. This sustained release effect could improve the bioavailability of GTP with slow and limited absorption. The individual AUC_{0-\infty} values for free GTP and GTP loaded albumin nanoparticles were studied to determine the relative bioavailability of GTP and the mean ratio was observed to be 1.74, which indicates nearly about 2 fold increase in bioavailability after loading in albumin nanoparticles (Table 4.21). Volume of distribution was observed to be higher in case of free GTP compared to loaded GTP. Higher volume of distribution provides a low initial drug concentration. Therefore, lower the volume of distribution, higher the concentration
of drug in plasma (Kumar et al., 1991). In our study, GTP loaded albumin nanoparticles had lower volume of distribution, indicating a higher concentration of GTP in plasma. Similarly, total drug clearance was found to be higher in free GTP compared to GTP loaded albumin nanoparticle. Decrease in the total GTP clearance in case of GTP loaded albumin nanoparticles, indicates the presence of more amount of GTP in the plasma and less excretion. These pharmacokinetic parameters such as MRT, half life of the drug, volume of distribution and total drug clearance play a vital role in determining the dosage and the dosage intervals for drugs. Therefore, this study on rabbit model, in conjunction with in vitro studies, clearly showed that the bioavailability of GTP could be increased and sustained release could be achieved on loading of GTP in albumin nanoparticles.

5.10. TUMOR INDUCTION AND TREATMENT (IN MICE MODEL) – EVALUATION OF BIOCHEMICAL PARAMETERS

One of the major applications of GTP is their use as an anti cancer agent. Several works have been carried out on determining the anticancer property of GTP. In our study, the effect of GTP loaded albumin nanoparticles on ascetic tumour was studied. Ehrlich’s ascetic carcinoma (EAC) cells were injected to Swiss albino mice for the development of ascetic tumour. Pre treatment and post treatment of unloaded albumin nanoparticles and GTP loaded albumin nanoparticles were administered. After treatment, blood was collected and plasma was separated and stored for further experiments. The antioxidant activities (catalase, SOD and LPO) were studied from the stored plasma samples (Table 4.22). Recent studies state that decrease in catalase activity or accumulation of hydrogen peroxide correlates with cancer metastasis (Tsai et al., 2014). Similar results were obtained in our study, where catalase activity was observed to be significantly lower in tumour mice compared to normal mice. Post treatment of unloaded albumin nanoparticles and GTP loaded albumin nanoparticles showed better results compared to pre treatment. Catalase activity was found to be significantly higher in GTP loaded albumin nanoparticles compared to unloaded albumin nanoparticles.

SOD activity in tumour cells, pre treated and post treated with unloaded albumin nanoparticles and GTP loaded albumin nanoparticles were studied. Literatures state
that EAC mitochondria have small amounts of SOD activity (Oberley and Buettner, 1979). Arsalan et al (2014), showed that SOD activity in tumour cells is much lower compared to normal cells. Our results corroborated with the above studies, as the EAC cells showed significantly lower activity compared to that of control (Table 4.22). In this study also, post treatment of GTP loaded albumin nanoparticles were more effective compared to pre treated groups (similar results for catalase and SOD activities). Pre treated and post treated groups with unloaded albumin nanoparticles were not significantly different.

Lipid peroxidation activity in all the groups was studied. The MDA levels were determined as oxidative stress markers. The MDA values in tumour cells are found to be significantly higher compared to normal cells (Arsalan et al., 2014). In our study also, MDA values of EAC cells were observed to be significantly higher than that of the control group (Table 4.22). In this case also, post treated groups exhibited better results compared to pre treated groups and GTP loaded albumin nanoparticles showed better results than unloaded albumin nanoparticles. Therefore, through these studies it was observed that GTP had better antioxidant activity and could be used as a source for the treatment of tumours.

Induction of apoptosis in ascetic tumour cells on post treatment with GTP loaded albumin nanoparticles were further confirmed through TUNEL assay. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is an established method for detection of DNA fragments. Cells containing fragmented nuclear chromatin exhibits dark staining, which is the characteristic of apoptotic cells. This dark staining is associated with chromatin condensation. In our study, cells post treated with GTP loaded albumin nanoparticles showed more number of darkly stained cells, which were similar to that of positive control when compared to that of cells pre treated with GTP loaded albumin nanoparticles (Figure 4.29 and Table 4.24). Pre and post treatment with albumin nanoparticles showed apoptosis to some extent, which might to be due to albumin induced apoptosis (Erkan et al., 2007). Therefore it could be said that GTP loaded albumin nanoparticles could induce apoptosis in tumour cells.

Level of expression of TNF α, IL 2 and IL 10 in ascetic cells pre and post treated with unloaded albumin and GTP loaded albumin nanoparticles were studied through
ELISA. It has been reported that in cancerous conditions, TNF α, IL 2 and IL 10 are being over expressed, precisely the levels being raised or elevated (Chan et al., 1993; Glezerman et al., 1998; Howell, 2000). Due to their elevated expression levels in cancerous conditions, these protein markers were chosen for this study. The influence of pre treated and post treated albumin and GTP loaded albumin nanoparticles on expression levels of these protein markers in ascetic tumour were studied using ELISA technique. The expression levels of the three protein markers in ascetic tumour were in accordance with earlier published results. On pre treatment and post treatment with albumin and GTP loaded albumin nanoparticles, significant decrease in expression levels were observed (Table 4.25). GTP entrapped albumin nanoparticles showed better results compared to unloaded albumin nanoparticles. Moreover, post treated group with GTP loaded albumin nanoparticles showed better results. These data indicate that GTP loaded albumin nanoparticles are able to decrease the expression levels of TNF α, IL 2 and IL 10 in ascetic tumours and could be used as a source for the treatment of ascetic tumours.

5.11. STABILITY OF GTP LOADED ALBUMIN NANOPARTICLES

Stability of GTP loaded albumin nanoparticles were studied by storing the samples at different temperatures (4°C, RT (25±2°C), 37°C and 45°C) for a period of 6 months. Particle size analysis and ζ potential analysis were carried out for the samples on a weekly basis for 3 months and once a month for the next 3 months. As the time increased, size of the particles also increased in all the temperatures (Table 4.26.1). No significant difference in the mean diameter of the particles was observed for those stored at 4°C and for that of RT. Size of the particles stored at other two temperatures was significantly higher. This increase in the size of the particles over a period of time might be due to aggregation of particles within the medium. Surface charge of the particles stored at different temperatures was also measured. It was observed that as the storage time period increased, there was a decrease in the surface charge of the particles (Table 4.26.2). After a period of 6 months, particles stored at RT were observed to be more stable than those stored at other temperatures. Therefore, through our study it could be said that lower or higher temperatures could change the conformation of the particles, thereby decreasing its stability. Since samples stored at RT showed better results with regards to size and charge, the surface morphology of
the particles via AFM and HR SEM were studied for samples stored at RT. Through AFM, the particle diameter was observed to be 87.3 nm, while through HR SEM, it was 61 nm (Figure 4.30 and 4.31). Spherical particles were observed in both the cases. No significant difference was observed in size and shape of the particles freshly prepared and those particles stored over a period of 6 months at RT. This study indicates that RT could be a better temperature for storing GTP loaded albumin nanoparticles.

Further, the stored samples were analyzed for their change in the functional groups through FTIR and XRD. FTIR analysis of the stored samples showed three extra peaks (3531, 3062, and 2872) compared to that of a freshly prepared sample (Figure 4.32). The presence of these peaks might be due to the rearrangement of the functional groups of GTP loaded albumin nanoparticles, which might be due to the change in the structural conformation of the nanoparticle. X ray diffraction studies also showed absence of three peaks (2θ=21.3, 31.7 and 46.6°) in the stored samples which were found in the freshly prepared samples (Figure 4.33). This also might be due to change in the structural conformation of the particles upon storage.

**In vitro** release studies in simulated conditions were carried out for the samples stored at 25±2°C to analyze any change in the release pattern of GTP. The simulated conditions were similar to that as previously described (Section 4.4.5). After a period of 24 h, a maximum of 41% and 60% of GTP was released in simulated conditions of fasted and fed states respectively (Figure 4.34). It was observed that on storage, an increase in the cumulative percentage of GTP release was observed (36 and 51% in fasted and fed states for fresh samples). Freshly prepared sample showed a maximum cumulative release percentage of 36% and 51% in fasted and fed state, while an increase in the cumulative percentage of GTP release was observed upon storage for a period of 6 months. The GTP release pattern before and after storage was Zero order kinetics, but the release mechanism was affected upon storage (anomalous for fresh sample and super case II transport for stored samples). Super case II transport, observed upon storage, might be due to the enzymatic degradation of the particles upon storage, resulting in polymer relaxation and polymer erosion (Arifin et al., 2006).
Effect of alcohol on GTP release for samples stored at different temperatures was analyzed. None of the samples showed dose dumping even after storage for a period of 6 months (Figure 4.35). The results obtained were similar to that of freshly prepared GTP loaded albumin nanoparticles. Results showed that as the concentration of alcohol in the release medium increased, an increase in the cumulative percentage of GTP release was observed. For the samples stored at 45°C, ~99% of the GTP release was observed by the end of 48 h, but no dose dumping was observed in any of the cases (any of the stored temperatures). Therefore, GTP loaded albumin nanoparticles were safe with regards to dose dumping even up to 6 months. Most of the release data were fitted to Zero order kinetics and the mode of drug release was observed to be anomalous (Table 4.28), which was similar to that of freshly prepared GTP loaded albumin nanoparticles.

Antioxidant activity of the released samples stored at different temperatures for a period of 6 months showed no significant difference compared to that of freshly prepared samples (Figure 4.46). Samples stored at 4°C and RT showed higher antioxidant activity compared to that of 37 and 45°C. All of the released samples even after 6 months showed potent antioxidant activity. From this stability study it was observed that even after storage for a period of 6 months, no significant difference was observed in any of the parameters studied except surface charge which was decreased significantly. Among all the samples stored at different temperatures, GTP loaded albumin nanoparticles stored at RT exhibited better results compared to other two storage temperatures tested. Therefore, through this study it could be said that, GTP loaded albumin nanoparticles could be stored at RT for a period of 6 months.