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7. SUMMARY AND CONCLUSION

Paclitaxel (PAC), isolated from *Taxus brevifolia*, is one of the most potent and commonly prescribed chemotherapeutic agent for the treatment of various cancers such as lung, ovarian, bladder, breast, and head-and-neck cancers. Restriction in the use of higher dose of PAC is due to its dose-limiting toxicity, whereas, lower dose leads to multifactorial chemoresistance by activating nuclear factor (NF)-κB, and upregulation of multidrug resistance-1 (MDR-1) gene, Akt and mitogen-activated protein kinase (MAPK). To encounter multidrug resistance (MDR) of PAC, curcumin (CUR) was judicially selected to combat most challenging aspects of cancer chemotherapy. CUR, a polyphenolic phytoconstituent from the rhizome of *Curcuma longa*, shows its pleiotropic therapeutic effect in cancer by modulating intracellular signaling pathways as it: (i) inhibits the activity of transcriptional factor nuclear factor kappa B (NFκB), (ii) down regulates the intracellular levels of three major ATP-binding cassette (ABC) drug transporters, P-glycoprotein (P-gp), multidrug resistance associated protein 1 (MRP-1) and breast cancer resistance protein (ABCG2). Its antiproliferative, antiangiogenic, antimetastatic and proapoptotic properties set it as a potent chemopreventive molecule. CUR reported its synergistic chemotherapeutic effects on promoting the apoptotic response of PAC. Thus, by co-administering PAC and CUR, various formulations were successfully developed to improve the bioavailability and therapeutic efficacy of PAC by enhancing the cytotoxicity in wild-type and resistant cells. However, both the anticancer agents, PAC and CUR are poorly soluble in water and, thus show very less oral bioavailability. To line the burden associated with less solubility, marketed formulation of PAC is composed of Cremophor® EL (polyethoxylated castor oil) and dehydrated alcohol. Despite this, we are encountered with unavoidable hypersensitivity reactions associated with Cremophor® EL. Regardless, cancer nanotechnology is ceaselessly searching for the development of Cremophor® EL-free formulations.

Nanotechnology offers several advantages in the field of clinical investigations either as a diagnostic or therapeutic agent. In the treatment of cancer, targeting the organ either by passive or active strategies can minimize serious side effects (associated with the anticancer drug) by reducing the dose of drug along with minimal bio-distribution.
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Eventually, over the past few decades, significant advances on various lipidic nanoparticles establish them as one of the promising delivery systems for poorly aqueous soluble drugs for cancer therapeutics. But, rapid clearance by reticular endothelial system (RES) upon parenteral administration of lipidic nanoparticles puts another challenge to navigate researchers for the discovery of novel formulation strategies. To confront this problem, PEGylated sterically-stabilized nanoparticles were developed with hydrophilic polymer “polyethylene glycol (PEG)”, which can prolong systemic circulation due to its stealth character for its preferential accumulation effectively in solid tumor microenvironment via the enhanced permeability and retention (EPR) effect through the passive targeting mechanism.

In chapter 3, we successfully obtained PAC and CUR loaded stabilized PEGylated lipidic nanocapsules (D-LNCs) by including poloxamer and MPEG\textsubscript{2000}-DSPE for i.v. delivery in Ehrlich Ascites tumor bearing mice. The PEGylated D-LNCs exhibited a sustained drug release pattern in \textit{in vitro} release studies. \textit{In vivo} pharmacokinetic studies showed a prolonged circulation time and slower clearance of D-LNCs as compared with Paclitec\textsuperscript{®}. Formulation tolerability studies supported the escalation of dose of D-LNCs to formulate a biocompatible, biodegradable and effective nano-carrier system suitable for chemotherapeutic drug delivery. Based on biodistribution studies and tumor inhibition study, it is possible to conclude that D-LNCs showed its targeting ability as well as significant antitumor efficacy \textit{in vivo}. The results together indicated that this hemo-compatible Cremophor\textsuperscript{®} EL free formulation might serve as potential nanocarriers for tumor specific cancer therapy by enhancing i.v. bioavailability of poorly water-soluble drugs without any hypersensitivity problems. Moreover, lyophilization increased the physicochemical stability of D-LNCs by incorporating 5\% (v/v) of sucrose as a lyoprotectant, which may allow long-term storage. Further, to confer industrial advantages, some experimental works are under progress in our laboratory for the validation of this research in large scale.

Super paramagnetic iron oxide nanoparticles (SPIONs; usually magnetite, Fe\textsubscript{3}O\textsubscript{4}, or maghemite, γ-Fe\textsubscript{2}O\textsubscript{3}) can be actively targeted to the specific organ by external magnetic field and associated properties, such as super-paramagnetism, high-field irreversibility,
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High saturation field, extra anisotropy contributions and biodegradability or clearance from the body, make them attractive candidates for *in vivo* medical applications. The last several years have witnessed an extensive research in the development of targeted delivery of chemotherapeutic agents employing SPIONs to the interstitial tumor microenvironment. SPIONs, due to their significant use in imaging and delivery techniques, now-a-days are being used for theranostic (therapeutic and diagnostic) purposes, where SPIONs can be used for both *in vivo* targeted drug delivery to the desired tissue of interest using an external magnetic field, and simultaneous tracking of the biodistribution of the particles. Due to their application in biology, medical diagnosis and therapy, a considerable number of SPIONs and many formulations have been approved for clinical use for magnetic resonance imaging (MRI) and therapeutic applications. Some of the examples of approved SPIONs include: Lumiren® being employed for bowel imaging, Feridex IV® for liver and spleen imaging, Combidex® for lymph node metastases imaging, and most recently, Ferumoxytol® for iron replacement therapy. Though progress in clinical applications of SPIONs has been slow, the results are promising towards effective treatment of a variety of diseases apart from localized tumors.

In chapter 4, the major formulation hurdles during the loading of curcumin into SPIONs i.e poor solubility in our stabilizer of interest (oleic acid) was solved by effectively nanosizing it through SAS process by optimizing SAS process parameters at mixing vessel pressure (P) at 14 MPa, mixing vessel temperature (T) at 40°C, CO₂ flow rate (CFR) at 40 g/min, and solution flow rate (SFR) at 0.5 mL/min, solution concentration (SC) at 0.5% (w/v) in acetone. CUR was effectively nanosized to ≈430.6±4.1nm and exhibited approximately 2.83-fold enhancement in oleic acid solubility as compared to native CUR. In conclusion, nanonization with SAS process could be a promising approach to improve the solubility of CUR by altering its physicochemical properties and surface composition, effectively. SAS is a one-step efficient unit operation to produce completely dry, nanosized organic solvent-free particles with more than 70% product yield justifying its industrial applications. Apart from that, since the whole process is operated in a closed environment, it can be applicable to photosensitive drugs like CUR. Current preclinical and clinical studies already establish CUR as an chemopreventive
molecule. Apart from that CUR is reported to possess P-glycoprotein (P-gp) inhibitory activities. So, by utilizing both anticancer and P-gp inhibitory activity, our research work structured further to load SAS processed CUR into SPIONs along with paclitaxel (PAC) to evaluate the anticancer activity in vivo.

In chapter 5, SPIONs were prepared by classical co-precipitation reaction and homogeneously dispersed SPIONs in aqueous medium (ferrofluid) were obtained by miniemulsion process, wherein surfactants were adsorbed at the interface between the organic phase and aqueous phase, and provided either an electrostatic or steric barrier between the organic droplets which prevented coalescence of the emulsion. SPIONs, which can be applied in biology, medical diagnosis and therapy, should be stable in water at neutral pH and physiological salinity. Such colloidal stability depends on two prime factors: (i) small dimensions of the particles to avoid precipitation due to gravity, and (ii) charge and surface chemistry, which give rise to both steric and coulombic repulsions. An extensive study was carried out for aqueous-phase transfer of oleic acid-coated SPIONs with respect to different pharmaceutical surfactants. Aqueous ferrofluid was found unstable with Span 20, Poloxamer 188 and Poloxamer 407 and vitamin E TPGS, whereas it was found to be stable with surfactants like SDS, Tween 20, Brij 35, Myrj 52 and docusate sodium. Pharmaceutical surfactants having low HLB values for example Span-20 (HLB; 8.6), and vitamin E-TPGS (HLB; 13.2), when used alone, can’t produce stable o/w emulsions, but they can be used in combination with varying proportions of high HLB surfactants to produce stable o/w emulsions. During the study, it was also found that some pharmaceutical surfactants, though having high-HLB value can’t support the formation of stable o/w emulsion for example Poloxamer 188(HLB;29) and Poloxamer 407 (HLB;18-23). Stable ferrofluid was developed by using high HLB value surfactants for example SDS (HLB; 40), Tween 20 (HLB; 16.7), Myrj 52 (HLB; 16.9), Brij 35 (HLB; 16-17) and docusate sodium (HLB; 32).Though vitamin E-TPGS, Poloxamer 188 and Poloxamer 407 have additional advantage of being P-gp inhibitors, they can’t be used alone for imparting stability to ferrofluid whereas Tween 20 , Myrj 52, and Brij 35 can be used as P-gp inhibitor to arrest tumor progression. In our experiment, we considered those formulations, in which particle sizes were less than 250 nm and which were found to be stable under accelerated stability studies for drug products.
intended for storage in a refrigerator. So, the formulation scientists may go ahead with stabilized ferrofluid for their pharmaceutical research in targeted drug delivery systems. Further, we have synthesized spherical and mono-dispersed CUR-loaded SPIONs by using oleic acid as a stabilizer and Myrj 52 as a surfactant. Apart from the anticancer activity of CUR, OA and Myrj 52 will also promote its anticancer activity by P-gp inhibition. During in vitro localization study of CUR-OA-SPIONs, the nanoparticles have been successfully localized at the side wall of the glass capillary with the use of a permanent magnet located close to the glass capillary. The size of the aggregate of CUR-OA-SPIONs increased gradually with time (t = 0+ s to t = 500 s), while no significant change in the size of the aggregate was observed after time t = 500 s).

In chapter 6, we successfully obtained and characterized PAC and CUR-loaded and Myrj 52 stabilized D-SPIONs for i.v. delivery in Ehrlich Ascites tumor bearing mice. Finally, we have compared in vivo therapeutic evaluation of D-LNCs and D-SPIONs with marketed formulation “Paclitec®”. From in vitro cell viability assay against MCF-7 and MCF-7/ADR cell lines, it was observed that anticancer activity of D-LNCs and D-SPIONs containing 0.0001-10 µM equivalent of PAC were dependent upon both concentration gradients of formulations, as well as incubation period during cytotoxic treatment. As compared to Paclitec®, our developed formulations are proved to be more cytotoxic against MCF-7 and MCF-7/ADR cell lines.

Comparative in vivo pharmacokinetic studies were carried out after a single dose intravenous (i.v) administration of D-LNCs and D-SPIONs having PAC equivalent of 15 mg/kg body weight in Ehrlich Ascites tumor (EAT)-bearing female Swiss Albino mice and compared with the same dose equivalent of marketed formulation “Paclitec®”. For magnetic drug targeting purpose, we placed a permanent magnet over the induced tumor site before the i.v. administration of D-SPIONs to anaesthetized mice. D-LNCs showed a prolonged circulation time and slower elimination compared with marketed formulation of Paclitec®. From the plasma concentration vs. time profile graph, i.v. bioavailability (AUC0-∞) of D-LNCs was found to be increased approximately 2.91-fold (p < 0.001) as compared to Paclitec®, whereas AUC0-∞ of D-SPIONs was found to be a little increased (1.18-fold; p < 0.001) as compared to Paclitec® treatment group. This might be due to
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PEGylation of D-LNCs, which favored long-circulation of D-LNCs, whereas for D-SPIONs, it could have been that D-SPIONs were attracted towards the tumor site due to the applied magnetic field which increased the bioavailability of loaded drug inside tumor microenvironment. Further, it was confirmed by biodistribution studies after a single dose intravenous (i.v.) administration of D-LNCs and D-SPIONs in tumor-bearing mice. Considering biodistribution studies, we observed significantly higher concentration of PAC in tumor tissue for D-SPIONs (9.31-fold) and for D-LNCs (4.64-fold) as compared to Paclitec®. This trend might be due to the localization/attraction of D-SPIONs from the systemic circulation towards tumor tissue by the applied magnetic field, which reduced PAC concentration in other major organs and minimized the chance of side effects.

Based on tumor inhibition study, it is possible to conclude that D-SPIONs, with magnetic field, showed its targeting ability, as well as significant antitumor efficacy in vivo compared to D-SPIONs without magnetic field. D-LNCs also showed better tumor-inhibition profile as compared to Paclitec®, but it was less effective in localized drug delivery as compared to D-SPIONs applied with magnetic field. Formulation tolerability studies and hemolysis studies concluded that these hemo-compatible Cremophor® EL- free formulations might serve as potential nanocarriers for tumor specific cancer therapy without any hypersensitivity problems. Moreover, accelerated stability studies for drug products intended for storage in a refrigerator allowed long-term storage of these formulations. Thus, this selective and enhanced tumor accumulation of D-LNCs and D-SPIONs validates our combined strategy of passive targeting (by EPR effect) and active targeting (by the application of external magnetic field) to target solid tumor microenvironment.