2. LITERATURE REVIEW

The cancer incidence rate in developing countries like India is reaching pandemic proportions as shown in Figure 2.1. That’s why the Indian Council of Medical Research (ICMR) has urged the Government of India to declare cancer a notifiable disease. Cancer is one of the leading causes of mortality in India, with about 2.5 million cancer patients, 1 million new cases added each year and with a chance of the disease increasing five-fold by 2025 (http://www.icmr.nic.in/ncrp/cancer_reg.htm). The causes of such high incidence rates of cancer may be due to internal factors (genetic, mutations, hormonal, poor immune conditions) and external or environmental factors (food habits, industrialization, over growth of population, lifestyle-related). There is a high probability of curing and preventing cancers if detected early in stage I or stage II which can lead a healthy life, but due to ignorance among public, delayed diagnosis and lack of adequate medical facilities mark cancer as a “killer disease”.

![Figure 2.1. Estimated incidence and mortality rates of cancer each year in India: Men and Women (Source: Globocan 2008).](http://www.icmr.nic.in/ncrp/cancer_reg.htm)
predominant killers. While observing, cancer scenario in India, the following Figure 2.2 shows how cancer cases have been progressively increasing from 2004 to 2015.

![Figure 2.2. Year wise total cancer prevalence in India [Source; ICMR, 2006; 2009].](image)

Based on the facts and figures from 2004 to 2015, predictions have been made for new cancer cases in 2020 which can be best suited by the following quote “the battle against cancer in the future is only going to get more and more monumental”.

### 2.1. SOLID TUMORS
Solid tumors are abnormal mass of tissue which may be benign (not cancerous), or malignant (cancerous). Different types of solid tumors (Figure 2.3) are named on the basis of their origin, such as carcinomas, sarcomas, and lymphomas. The term “tumor” and “cancer” should not be confused as the word tumor does not always imply cancer. Further, a solid tumor is often used to distinguish a localized mass of tissue from leukemia, which mostly affects the blood.

I. **Carcinomas**- originate from body’s glandular cells and epithelial cells, which line body tissues.

II. **Sarcomas**- originate from connective or supporting tissues, such as bone or muscle.

III. **Lymphomas**- originate from lymphoid organs, such as the lymph nodes, spleen, and thymus, which produce and store infection-fighting cells.
2.2. TREATMENT STRATEGIES FOR SOLID TUMORS

The best approach to treating cancer provides a balance between therapeutic effectiveness and minimization of treatment-associated side effects, which includes a combination of surgery, radiation therapy, and chemotherapy.

I. Surgery

Excision of tumor, along with a margin of normal tissue surrounding the tumor is most successful, when tumor is identified in early stage. The risk of local recurrence due to infiltration of malignant cells is thus reduced. But limitation of surgery is associated with most metastatic diseases, hematological malignancies and patients with co-existing systemic diseases (diabetes, cardiovascular disease, etc.). Sometimes, extensive surgery may lead to significant deformity or organ dysfunction.

II. Radiation therapy

Ionizing radiation’s energy triggers apoptosis and cell death either by breaking DNA strands or by generating hydroxyl free radicals in presence of water molecules that can further damage DNA. Radioisotope preparations administered intravenously can target specific tissues, such as iodine (\textsuperscript{131}I) for thyroid malignancies, Strontium (\textsuperscript{89}Sr) and Samarium (\textsuperscript{153}Sm) for metastatic bone tumors. But limitations of radiotherapy lie with the
potential toxic effects, such as bone marrow suppression, xerostomia, inflammation, and other radiation injuries.

III. Chemotherapy
Most chemotherapeutic agents target malignant cells, either by directly damaging DNA and/or interfering with cell division and/or activating programmed cell death (apoptosis). Some anticancer molecules either interfere with mitosis or block the utilization of nucleotides required for DNA synthesis.

2.3. LIMITATIONS OF CURRENT CHEMOTHERAPEUTICS APPROACH

I. Treatment-related systemic toxicities
Chemotherapy carries a high risk, and the more effective drugs tend to be more toxic. Most chemotherapeutic agents are highly toxic and have limited-tumor specificity. This results in a relatively narrow therapeutic window, where the maximum tolerated dose is limited by dose-dependent toxicity. Depending on the choice of drugs, different organs or tissues can be irritated or damaged by the non-specific action of the cytotoxic agents. While side-effects such as nausea, vomiting, fatigue, hair loss are commonly caused by almost all cytotoxic drugs, some side-effects are drug specific for e.g., cardio-toxicity observed during anthracyclines therapy (Kalyanaraman et al., 2002).

II. Lack of target specificity
Conventionally administered cytotoxic agents often extensively and indiscriminately bind tissues and serum protein in a highly unpredictable manner. Many current therapies are administered into the bloodstream and rely on the leaky vasculature of tumor tissue to accumulate the drug within the cancer tissue. Only a small fraction of the drugs reach the tumor site, which in turn leads to reduced therapeutic efficacy and increased systemic drug toxicity.

III. Poor drug solubility
Most anticancer drugs, especially those with excellent anticancer effects, such as taxanes (paclitaxel and docetaxel), camptothecins (topotecan and 9-aminocamptothecin),
topoisomerase-II inhibitors (etoposide and teniposide), the anthracylines (doxorubicin, epirubicin and daunorubicin), all Vinca alkaloids (vincristine, vinblastine, vinorelbine), ifosfamide and mitoxantrone have low oral bioavailability due to limited aqueous solubility. Adjuvants, such as emulsifiers, have to be used for the clinical administration of these drugs, are themselves are associated with serious side effects, some of which are life threatening.

IV. Development of drug resistance

Drug resistance may evolve during the course of treatment as the cancer progresses, and is often associated with a group of membrane proteins or ATP (adenosine triphosphate)-binding cassette (ABC) proteins. It is the main cause of treatment failure in cancer as it leads to recurrence or even death. Most importantly, P-glycoprotein (P-gp) mediated efflux of the diffused drug is the major concern in multi drug resistance (MDR) (Aszalos and Ross, 1997; Szakács et al., 2006).

Various processes play a vital role during drug delivery to solid tumors, which involve mainly (i) transport within a vessel (for example, blood circulation), (ii) transport across vasculature walls into surrounding tissues, and (iii) transport through interstitial space within a tumor. And these processes are dependent upon either physicochemical properties of a drug molecule (for example, molecular or particle size, drug binding to cellular macromolecules, diffusivity) or the biologic properties of a tumor (for example, tumor vasculature, tumor cell density, extracellular matrix components, interstitial fluid pressure (IFP), tissue structure and composition), etc. In the last 2-3 decades, the role of efflux proteins and membrane transport in tumor drug delivery has also been evaluated (Jain and Stylianopoulos, 2010; Jang et al., 2003).

Thus, cancer drug delivery is no longer simply wrapping the drug in the new formulations, but targeted delivery approach clubbed with nanotechnology for the effective delivery of cytotoxic drugs to the malignant tumors to overcome lacunas in conventional delivery approaches employed in chemotherapy.
2.4. SELECTION OF DRUG

2.4.1. Paclitaxel (PAC): A chemo-therapeutic molecule

Paclitaxel (PAC), a mitotic inhibitor isolated from the bark of Pacific Yew (*Taxus brevifolia*) was first discovered by Monroe E. Wall and Mansukh C. Wani in 1967 and named as Taxol™. It was later discovered that Taxol™ was produced by a fungal endophyte, isolated from the phloem tissue of the Pacific Yew tree (Stierle et al., 1993). For the first time, it was commercially developed by Bristol-Myers Squibb Company under the trade name of Taxol™. It is a white crystalline powder, white to off-white in appearance having the melting point around 216-217°C. It is a lipophilic molecule of a molecular weight of 853.9 Da with empirical formula of C₄₇H₅₁NO₁₄ (Figure 2.4).

![Figure 2.4. Chemical structure of paclitaxel (PAC).](image)

**Systematic (IUPAC) name**: (2α,4α,5β,7β,10β,13α)-4,10-Bis(acetyloxy)-13-{{[(2R,3S)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate.

It is one of the most effective chemotherapeutic drugs and is mainly used to treat lung, ovarian, bladder, breast, and head-and-neck cancers, etc (Crown and O'Leary, 2000). PAC targets tubulin, where beta-tubulin subunit is known to have the binding site for PAC (Jordan and Wilson, 2004). The mechanism of action of PAC is to protect and stabilize microtubules against disassembly and inhibit late G₂ or M phases of cell cycle, thereby causing cell death.

Due to aqueous solubility limitations i.e. ~0.4 μg/mL, marketed formulation of PAC is composed of polyoxyethylated castor oil (Cremophor® EL) and dehydrated ethanol (50/50, v/v) under the trademark “Taxol™”. But the vehicle Cremophor® EL is associated with serious side effects, such as hypersensitivity reactions (Gelderblom et
al., 2001). Thus, prolonged infusion time and premedication are essential. Co-delivery of PAC with Cremophor® EL are always associated with unpredictable non-linear plasma pharmacokinetics as Cremophor® EL alters the pharmacokinetic profile of PAC in vivo (Sparreboom et al., 1996). PAC can also induce drug resistance as it is a substrate of P-glycoprotein (P-gp) molecule, which actively effluxes PAC out of the cells (Gallo et al., 2003). To encounter this problem, co-formulation of PAC and several P-gp inhibitors, such as verapamil (Berg et al., 1995) and PSC 833 (Fracasso et al., 2000) were administered, but they still disappoint by altering the pharmacokinetics and biodistribution of PAC. NP-assisted chemotherapeutic drug delivery systems are promising vehicles, as they enhance aqueous solubility as well as therapeutic effectiveness of PAC. Abraxane®, a PAC albumin-conjugated NP with a mean particle size of ~130 nm, was approved by the FDA in 2005 for the treatment of metastatic breast cancer. Also, they show negligible toxicity as compared to Taxol™, which can be administered within 30 min without any pretreatment. But, therapeutic response of Abraxane® was silent with respect to improvement in survival rate and P-gp-mediated drug resistance, which potentiates researchers to develop alternative PAC formulations.

2.4.2. Rationale behind selecting paclitaxel as model anticancer drug

Taxanes such as PAC was selected as model drug candidates for targeted drug delivery system because of the following properties:

- Potent and most widely used anticancer drugs in various malignancies.
- Low aqueous solubility and low permeability, belongs to BCS class IV.
- Poor oral bioavailability (1-8 %).
- Hypersensitivity reactions associated with the currently-marketed formulations of Taxol™.
- Patient non-compliance.
- Risks of infection and extravasations that are associated with intravenous access lines.

Various nanoparticles (NPs) for the delivery of PAC such as lipid-based NPs, polymeric NPs, polymer conjugates, metallic NPs, inorganic NPs, carbon nano tubes, nanocrystals, cyclodextrin NPs, etc. were addressed well in Table 2.1.
Table 2.1. Various nanoparticulate systems of paclitaxel (PAC)

<table>
<thead>
<tr>
<th>Types of NPs</th>
<th>Name of polymer</th>
<th>Modification/ conjugation/</th>
<th>Preparation method</th>
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<td>Polymeric nanoparticles</td>
<td>PAMAM</td>
<td>Ester / succinic acid</td>
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<td>Ester / FITC-folic acid</td>
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<td>Heparin</td>
<td>Ester / valine, leucine, phenylalanine</td>
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<tr>
<td></td>
<td>Ester / folic acid</td>
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<td>in vivo</td>
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<tr>
<td>PGG</td>
<td>Ester</td>
<td>-</td>
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<td>(Van et al., 2010)</td>
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<td></td>
<td>Ester / PEG-RGD</td>
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<td>HA</td>
<td>Ester / valine, leucine, phenylalanine</td>
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<td></td>
<td>Oligomer</td>
<td>Dialysis</td>
<td>in vitro</td>
<td>(Saravanakumar et al., 2010)</td>
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<tr>
<td>HPMA</td>
<td>Ester / Gly-Phe-Leu-Gly</td>
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<td>PG</td>
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<td>Emulsion-solvent evaporation</td>
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<td>Modified solvent displacement</td>
<td>Emulsion and evaporation</td>
<td>Co-solvent extraction</td>
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<td><strong>TPGS</strong> (emulsifier)</td>
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<td><strong>DLPC</strong> (emulsifier)</td>
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<td><strong>DPPC</strong> (emulsifier)</td>
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<td><strong>Chitosan</strong></td>
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<td><strong>DMAB</strong></td>
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<td><strong>MMT</strong></td>
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<td><strong>MMT, HER2 (targeting)</strong></td>
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<td><strong>RGD (targeting)</strong></td>
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<td><strong>Pluronic P85, transferrin (targeting)</strong></td>
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<td><strong>PCL</strong></td>
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<td><strong>PCL-pluronic F68</strong></td>
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<td><strong>PCL-pluronic F68, DMAB</strong></td>
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<td><strong>mPEG-PCL, Angiopep (targeting)</strong></td>
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<tr>
<td><strong>mPEG-PCL</strong></td>
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<tr>
<td><strong>PEG-PCL</strong></td>
<td><em>in vivo</em></td>
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*(Mu and Feng, 2003)*
*(Liu et al., 2010)*
*(Feng and Huang, 2001)*
*(Kim et al., 2008a)*
*(Bhardwaj et al., 2009)*
*(Dong and Feng, 2005)*
*(Sun et al., 2008)*
*(Wang et al., 2011b)*
*(Shah et al., 2009)*
*(Devalapally et al., 2007)*
*(Ma et al., 2010)*
*(Mei et al., 2009)*
*(Xin et al., 2010b)*
*(Wang et al., 2011a)*
*(Forrest et al., 2008)*
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<td>PCL-PEEP, galactosamine, (targeting)</td>
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<td>PLA-PEG-PLA, PEG-PLA-PEG</td>
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<td>Glyceryl monooleate</td>
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<td>surfactants (dextran 70, cholesterol, PVA, and lecithin)</td>
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<tr>
<td>folic Acid (targeting)</td>
<td>Desolvation</td>
<td>in vivo</td>
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<td>Octaldehyde</td>
<td>Dialysis</td>
<td>in vitro</td>
<td>(Gong et al., 2009)</td>
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<tr>
<td>PEG-PE</td>
<td>EPC, solid triglycerides, cationic Lipofectin lipids</td>
<td>Solvent evaporation</td>
<td>in vivo</td>
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### Liposomes

| Phosphatidyl choline, cholesterol, cardiolipin | - | in vitro                  | (Zhang et al., 2005) |
| HSPC, cholesterol, PEG-DSPE | - | in vivo                  | (Yoshizawa et al., 2011) |
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#### Solid lipid nanoparticles

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<td>Tripalmitin, Epikuron 20, butanol, Na-taurocholate, cholesteryl hemisuccinate</td>
<td>-</td>
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<tr>
<td>Stearic acid, lecithin, Brij 78 (or pluronic F68 and DSPE-PEG)</td>
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<tr>
<td>Trimyristin, EPC, PEG2000-PE</td>
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<tr>
<td>Glyceryl palmitostearate (or glyceryl monostearate), Poloxamer 407</td>
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<td>in vitro</td>
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<tr>
<td>Lipoid S 100, Sucrose fatty acid esters</td>
<td>-</td>
<td>in vitro</td>
<td>(Arica Yegin et al., 2006)</td>
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<tr>
<td>Stearylamine, soya lecithin, poloxamer 188</td>
<td>-</td>
<td>in vivo</td>
<td>(Pandita et al., 2011)</td>
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<tr>
<td>Monostearin, stearic acid, Glycerol tristearate, ATO 888</td>
<td>-</td>
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<tr>
<td>Emulsifying wax, Brij 78</td>
<td>-</td>
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<td>Glyceryl tridodecanoate, Brij 78</td>
<td>-</td>
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#### Lipid nanocapsules

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<tr>
<th>Composition</th>
<th>Platform</th>
<th>Study Type</th>
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<td>Labrafac®, Lipoïd® S75-3, Solutol® HS15</td>
<td>-</td>
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<tr>
<td>Captex® 8000, Lipoïd® S75-3, Solutol® HS15</td>
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<td>Miglyol 812, Brij 78, TPGS</td>
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<td>Lipiodol, PEO-PPO-PEO, functionalized PEG, Folic acid (targeting)</td>
<td>-</td>
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</table>
### Lecithin, Dynasan 118, Miglyol 812, folate-PEG-PE, Span 60 (or PEG PE)
- *in vivo*  
  (Jores et al., 2004)

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- *in vivo*  
  (Wang and Ho, 2010)

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  (Constantinides et al., 2000)                                              |
| Oil blend, EPC, Tween 80, glycerol                                          | -                    | *in vivo*  
  (Kan et al., 1999)                                                          |
| Egg lecithin, flaxseed oil, DSPE-PEG2000                                    | -                    | *in vitro*  
  (Ganta and Amiji, 2009)                                                     |
| Lecithin, butanol, myvacet (or capmul, myvacet)                              | -                    | *in vivo*  
  (Nornoo et al., 2008)                                                       |
| Pine nut oil, egg lecithin, stearylamine, deoxycholic acid                  | -                    | *in vivo*  
  (Tiwari and Amiji, 2006)                                                    |
2.4.3. Curcumin (CUR): A chemo-preventive molecule

Chemoprevention is a promising anti-cancer approach, which uses natural or synthetic chemicals that allow suppression, retardation or inversion of carcinogenesis (Kelloff et al., 1994). Most chemo-preventive agents either act as blocking agents by preventing the initiation step of carcinogen activation or as suppressing agents, by inhibiting malignant cell proliferation during promotion and progression steps of carcinogenesis (Duvoix et al., 2005). Extensive research over the last half century has revealed CUR as one of the most studied chemo-preventive and chemo-therapeutic agents.

![Chemical structure of curcumin (CUR)](image)

**Figure 2.5. Chemical structure of curcumin (CUR).**

**Systematic (IUPAC) name:** (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione

CUR, a natural diphenol (Figure 2.5), extracted from the rhizomes of Curcuma longa, is traditionally used as an ayurvedic medicine for the prevention of various clinical disorders like Alzheimer's disease, arthritis, diabetes, HIV replication, myocardial infarction, and wound healing, etc (Aggarwal and Sung, 2009; Boaz et al., 2011; Bright, 2007; Gregory et al., 2008; Henrotin et al., 2010; Mishra and Palanivelu, 2008). But in the last decade, CUR had been the major focus of research for the prevention and treatment of various cancers, such as melanoma, head and neck, breast, colon, pancreatic, prostate and ovarian cancers (Aggarwal et al., 2003; Lin et al., 1997; Lin et al., 1998; Mahady et al., 2001; Wilken et al., 2011). Numerous research teams have proved effectiveness of CUR as a potent anticancer molecule in some tumors, chemically induced by benz(a)pyren, 7,12-dimethylbenz(a)anthracene or phorbol esters.
(Azuine and Bhide, 1992a, b), and N-methyl-N0-nitro-N-nitrosoguanidine (MNNG) (Ikezaki et al., 2000).

CUR-induced apoptosis is activated by Akt dephosphorylation, Bcl-2, Bcl-XL and inhibitor of apoptosis (IAP) protein inhibition, as well as cytochrome c release and caspase 3 activation (Figure 2.6). But in some cases, apoptosis is found to be caspase 3 independent and increase of glutathione (GSH) levels blocked its activation (Piwocka et al., 2002; Piwocka et al., 2001; Piwocka et al., 1999). Also CUR was found to inhibit proteasome activity in mice causing apoptosis through caspase 9 activation.

Further inhibition of vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2 in EAT cells, VEGF and angiopoietin 1 inhibition in NIH 3T3 cells, and tyrosine kinase Flk-1/KDR (VEGF receptor-2) in HUVEC cells establish it as anti-angiogenesis agent (Arbiser et al., 1998; Gururaj et al., 2002). CUR also reduces telomerase activity by inhibiting human telomerase reverse transcriptase (hTERT) expression in MCF-7 cells.
(Ramachandran et al., 2002). As compared to chemotherapeutic molecule, CUR does not impart any cytotoxic effects on healthy cells while inducing apoptosis in various cell lines including HL-60, K562, MCF-7 and HeLa (Roy et al., 2002). Upon administration of higher doses of CUR (8 g/day), it did not represent any cytotoxicity in patients (Cheng et al., 2000).

2.5. CANCER TARGETED NANOPARTICLES: TARGETING OVERVIEW AND RECENT ADVANCEMENTS

Conventionally, the first-line treatment of solid tumors is their surgical removal followed by a regimen of chemotherapy and/ or radiation treatment. Unfortunately, these strategies often fail and the patient may discontinue the treatment before the complete eradication of tumor due to several severe side effects (Brannon-Peppas and Blanchette, 2012). Presently, a variety of anticancer agents are available belonging to different categories like plant origin, hormones, enzymes, biologicals, semi-synthetic and synthetic molecules. However, they have certain therapeutic and toxicological limitations. Intracellular transport of therapeutically active substances, particularly in cancerous cell, is a potentially challenging task for the drug delivery systems. Distribution of drugs within the cellular and tissue structure essentially depends upon its physiochemical properties like pKa, hydrophilicity, polarity and electrostatic charge. However, all these criteria do not necessarily fit in the characteristics of tumor cell because drug can be distributed towards healthy tissue rather than the target (Brigger et al., 2002). In addition, most of the anticancer drugs are not soluble in water or physiological solution, thus necessitating the use of pharmaceutical solvents for the administration of drugs causing life threatening effects (Torchilin, 2004). Consequently, conventional chemotherapy for systemic delivery of chemotherapeutic agents often fails due to inadequate delivery of the drug to target cell/ tissue and has a tremendous impact on reducing the quality and expectancy of life. Other disadvantages that are associated with current conventional anticancer therapy and imaging technology are inefficient cellular uptake, uptake by the immune system (RES), mononuclear phagocyte system and accumulation in non-targeted organs (Zahr and Pishko, 2009). Therefore, the urgent need in the treatment of tumor therapy is the designing of a delivery system that can selectively deliver the anticancer agents to the target tissue with highly localized
bioavailability, thereby achieving therapeutic efficacy while minimizing side effects. Novel drug delivery systems, such as nano-sized carriers have shown to improve the treatment of cancer with reduced dosing frequency and lesser side effects (Allen, 2002). Advancement in nanotechnology-based delivery approaches and imaging techniques have a growing interest in nanomedicine based research. For example, nanoparticle formulations, such as Doxil® and Abraxane®, have demonstrated clinical significance by increasing drug efficacy and diminishing toxicity (Malam et al., 2009; Miele et al., 2009). Need for nanotechnology based drugs and drug delivery devices will grow by more than 17% annually reaching an approximately $53 billion market in 2011 and an estimated $18 billion in 2014 (Allen, 2002). Moreover, the US National Science Foundation predicts that half of the pharmaceutical industry product line will comprise of central nanotechnological design features by 2015. At least 12 nanotechnology based medicines are already approved and progressively more are seen entering the active developmental stages (Kim and Lim, 2002). Hence, a steady succession of new nanomedicine, imaging, and diagnostic agents are anticipated to seek (and possibly gain) regulatory approvals and subsequent access to human use.

2.5.1. Site-specific drug delivery and biological contemplation

Ideally, for effective drug targeting, it is essential that a drug should not eliminate quickly and the drug carrier should provide a pharmacokinetic profile that will allow the drug to interact with its target (Zahr and Pishko, 2009). Therefore, in the designing of targeted nanoparticle, the painstaking lessons learned from the polymer based and liposomal drug delivery systems must be taken into consideration. Since, it is established now that upon intravenous injection the unprotected liposomal and polymer based drug delivery systems are rapidly cleared from the blood by reticuloendothelial system (RES) and gets accumulated in the liver conditioning their rapid first pass metabolism from the systemic circulation followed by metabolic degradation and excretion. This is an imperative consideration while designing targeted nanoparticles intended for cancer therapeutics targeting cells located nearby the mononuclear phagocyte system. The performance of nanoparticles inside the vascular compartment is controlled by a complex array of factors such as shape, density, size distribution, surface characteristic, zeta potential, magnetism, reactivity and release characteristics of nanoparticles. All these factors control the flow
properties of nanoparticles, bifurcation in vascular compartment as well as modulation of circulation time and mode of entry into the cell (Yvonne and Thomas, 2010).

2.5.2. RES (Reticuloendothelial system) escaping

A major barrier that a drug delivery system must be able to avoid in the systemic circulation is the removal of delivery system or drug by phagocytic cells of mononuclear phagocytes system (MPS). Since the nanoparticles are usually taken up by the liver, spleen and other parts of the reticuloendothelial system (RES) depending on their surface characteristics and undergo opsonisation in the blood and clearance by RES (Brigger et al., 2002), the nanoparticles, therefore should be designed to avoid these interactions and their possible clearance from the vascular compartment, particularly opsonisation process. Opsonization is the process by which a pathogen, foreign particle or particulate drug carrier is marked for ingestion and destruction by a phagocyte. It involves the binding of an opsonin i.e. antibody to the surface of foreign particle due to which phagocytes are attracted towards it. Finally, the foreign particle is engulfed and digested by lysosomes. For example, when normal colloidal gold nanoparticles were intravenously injected into a mouse, it was observed that 95% of gold nanoparticles were cleared from the vascular compartment within 10 min (Paciotti and Tamarkin, 2007), indicating the need of suppression of opsonisation, avoiding MPS recognition and receptor mediated phagocytosis while the designing of targeted nanoparticle. A newer practical approach to avoid RES uptake of nanoparticles is the modification of nanoparticle surface. In such cases, increasing surface hydrophilicity i.e. adding a hydrophilic polymer coat to the metallic nanoparticle carrier, will make them imperceptible to the RES and would thus reduce opsonisation and suppress macrophage recognition. This coating is referred to as stealth. Most commonly used stealth agents for this purpose are polyethylene glycol (PEG), poloxamers, carbopols and block copolymers (Paciotti and Tamarkin, 2007). It has been established for PEG that it contains high concentration of hydrated groups which sterically inhibit the interaction with blood-born opsonins. Intravenous injections of sterically stabilized nanoparticles result in prolonged circulation time and accumulation in tumor. Another factor affecting the opsonisation process are physicochemical properties of nanoparticles like surface characteristics (size, surface charge), bio-distribution and residence times of these particles in vivo. It is commonly
seen that neutral systems tend to remain longer in blood circulation but their charged counterparts are cleared by RES readily (Brannon-Peppas and Blanchette, 2012; Paciotti and Tamarkin, 2007). Furthermore, particle size of around 1-2 micron undergoes phagocytosis and higher size i.e. around 6 micron is trapped in pulmonary capillaries (Yvonne and Thomas, 2010). Therefore, it is postulated that, to avoid clearance by RES, the NPs should be formulated of not more than 100nm in size and should have sterically stabilized neutral surface.

### 2.5.3. Passive tumor targeting

In passive targeting, the bio-distribution of NPs is mediated by their physiological conditions. In such approaches, we take the advantage of pathological condition of tumor cells (pH condition, temperature and specific enzymes) to allow accumulation of drug carrier at the target site. Enzymes such as alkaline phosphatase and plasmins are present in higher amounts at the tumor site. When the volume of tumor becomes 2mm$^3$, it becomes diffusion limited, which is overcome by angiogenesis (growth of new capillary in cell/ tissue) (Brannon-Peppas and Blanchette, 2012). In tumor cells, the characteristic features of angiogenesis are its aberrant tortuosity and abnormalities in basement membrane (Baban and Seymour, 1998). Since, tumor cells generally lack the well-defined lymphatic system as a result of which the interstitial pressure is maximum at the centre of tumor. These incomplete vasculatures of tumors result in leaky blood vessels (capillary). This hyper permeability of tumor vasculature is a key feature in passive targeting of drug carrier (Folkman et al., 1971; Hobbs et al., 1998). Due to leaky vasculature and poor lymphatic drainage, the drug carriers get trapped in the tumor vasculature and release the loaded drug. Figure 2.7 represents the capillary with Enhanced Permeability and EPR effect at tumor sites. However, this EPR is associated with accumulation of nanoparticles in blood capillaries near to healthy cell and can lead to recognition by MPS (Stolnik et al., 2012).
2.5.4. Active tumor targeting

Since passive targeting does not necessarily assure the internalization of nanoparticles by the targeted cell, therefore nanoparticles are modified with molecular targeting ligands for active targeting (Figure 2.8). Active targeting of NPs involves the interaction between peripherally conjugated targeting moiety and a corresponding receptor to facilitate the targeting of a carrier to a specific malignant cell (Sinha et al., 2006). It is now established that tumor cells over-express certain specific surface receptors, aptamers (Yigit et al., 2007), proteins and antibodies (Allen, 2002) which can be targeted for effective delivery of anticancer agents, facilitating the active targeting of nanoparticles. The bioconjugation of ligands, like monoclonal antibodies, proteins or peptides to the nanocarrier surface has been exploited in many cases for the purpose of concentrating therapeutic action to specific tumor cell through nanoparticles. These ligands (antibodies, saccharides, aptamers, hormones, lectin) bind to their specific receptor on the cellular surface and trigger the internalization process of drug delivery so that the anticancer drugs act on cellular organelles (e.g., mitochondria, microtubules, nucleus, etc.) by mean of receptor mediated endocytosis (RME). Several over-expressed growth factor receptors
have been used for selectively targeting the cancer cell. The description of many of these
targets has been reviewed (Ahmad et al., 2010; Carter et al., 2004).

**Figure 2.8.** Schematic representation of theranostic molecules conjugation to
tMNPs as targeted tumor imaging, sensing and therapeutics (Akhter et al., 2011).

Many pathological areas e.g. human ovarian carcinoma shows a distinct hyperthermia
around 42 °C. Some polymers with a low critical solution temperature (LCST) usually
precipitated at the temperature, above LCST. By this mechanism, the nanocarrier
structure is damaged and the drug is released inside tumor microenvironment. Apart from
that, local heating of the tumor can be achieved by various least invasive, easiest and
cheapest mean e.g. ultrasound. Bu using this temperature some nanocarrier systems
containing thermo-responsive polymers e.g. poly(N-isopropylacrylamide) (NIPAM) are
most widely in targeted drug delivery systems (Kono et al., 2002). Magnetic
nanoparticles either by the influence of external magnetic field or by conjugating with
targeting ligands can be guided to a particular target area via passive targeting (McBain
et al., 2008). Also due to increase in local temperature by using SPIONs in an alternating
magnetic field, allows for the elimination of the tumor, which based on the principle of
“magnetic thermal ablation” (Hilger et al., 2002). Some photo-responsive polymers,
when exposed with light of the appropriate wavelength, they tend to change their
properties, typically, the light may induce some structural transformations of specific
functions of this type of polymers. For example, cleavage of the pyrenyl-methyl esters caused the transformation of pyrene-containing hydrophobic methacrylate units to hydrophilic methacrylic acid units, when exposed to UV irradiation, resulting in micelle dissociation (Ghosh et al., 2009). But limitation with UV and visible light is that this wavelength is readily absorbed by the skin. For this infra-red or near infra-red light sensitive polymers are now extensively studied (Jiang et al., 2006). Drug release from the nanocarriers to a target area may also be triggered by external ultrasound. Induction of thermal or mechanical effects such as transient cavitation and or local heating due to high-intensity focused ultrasound (HIFU) causes phase transition of the polymers, which involves the drug release from nanocarriers (Schroeder et al., 2009).

2.6. PEGylation: A SUCCESSFUL PASSIVE TARGETING STRATEGY
Poly ethylene glycol (PEG) is a synthetic polymer of ethylene oxide, with the chemical formula of HO–(CH₂CH₂O)ₙ –H. Low molecular weight (< 500 Da) PEGs are colorless, viscous liquids, whereas higher molecular weight PEGs are waxy solids crystals having melting points increase towards an upper limit of 70°C (Bailey, 2012). PEG is soluble in water as well as in most common solvents including methanol, benzene, and dichloromethane, which makes easier use for pharmaceutical applications. PEGs are eliminated by a simple elimination pathways combined of renal (Andus and Raub, 2012) and hepatic clearance (Webster et al., 2007). PEG shows the lowest level of protein or cellular adsorption as compared to any polymer. Apart from that, it is nontoxic, non-antigenic, non-immunogenic, and has been approved by FDA for many injected biotech products. It can be available in various molecular weights and functionalized architectures (e.g., amino-, carboxyl- and sulfhydryl-terminated) to covalently attach to the surface available groups on the conjugating molecule (Roberts et al., 2012).

Upon systemic administration, most of NPs are cleared from circulation by the phagocytes from reticuloendothelial systems (RES). The process of opsonization, which reduces the circulation time of the NPs largely depend upon surface structure and hydrophilicity/hydrophobicity state of NPs. PEG prevents recognition by monocytes and macrophages, thus increases the circulation time by reducing this opsonization process,
allowing the NPs to remain longer in the blood pool (van Vlerken et al., 2007). The commonly used lipid derivative of PEG is methoxy-PEG-DSPE with a methoxy terminal which can prolong liposomes circulation time. But the methoxy terminal is too inactive to react with the ligands in mild conditions, so it is necessary to modify PEG-DSPE terminal mainly consisting of the hydroxyl groups to improve physicochemical properties of the polymers for targeted drug delivery (áO’Donoghue, 2010; Su et al., 2008). The common modification forms of PEG-DSPE derivatives include carboxylation, amination, and maleylation.

I. Carboxyl-terminated PEG-DSPE
Carboxyl groups are introduced to the terminal groups of PEG-DSPE block copolymers for easy reaction with the ligands for active target cells or tissues, such as transferring and peptide (Ishida et al., 2001; Su et al., 2008).

II. Amino-terminated PEG-DSPE (amino-PEG-DSPE)
One end of the PEG is selectively protected by protective groups such as fluorenyl-methyloxy-carbonyl and butyl-oxycarbonyl (Boc) for introducing amino group in the other end that reacts with DSPE.

III. Hydrazide-terminated PEG-DSPE (Hz-PEG-DSPE)
Ligands can also be covalently bound to the hydrazide groups grafted onto PEG-DSPE to form a hydrazide bond which has been successfully utilized to synthesize various ligand-modified PEG-DSPE copolymers for targeting drug-delivery systems (Cuong and Hsieh, 2011; Koning et al., 2002).

IV. Maleimide-terminated PEG-DSPE (Mal-PEG-DSPE)
Recently, PEG-DSPE modified with a maleimide group at the distal terminal of the PEG chain has now been widely used in targeted drug delivery systems due to its convenient and rapid reaction with ligands such as antibody and peptide (Kibria et al., 2011; Lu et al., 2011a).
A number of clinical trials involving PEGylated polymer–drug conjugates already showed promising results and few of them are successfully commercialized also (Table 2.2). PEGylation could also be used to improve the physicochemical characteristics of commonly used anticancer drugs, like paclitaxel, docetaxel, and camptothecin, which have poor aqueous solubilities along with serious side effects and require frequent administration. In addition, the vasculatures of cancer tissues which can cause the accumulations of supramolecular entities like polymers and liposomes due to EPR effect (Maeda et al., 2000). Thus, it is hoped that PEGylated lipidic nanoparticles encapsulating anticancer drug molecules will selectively accumulate in cancer tissues and that will lead to sustained drug release by passive targeting mechanism as described in the section 2.5.3. In other aspects, due to some shortcomings of different passive targeted NPs for drug delivery, superparamagnetic iron oxide nanoparticles (SPIONs) constitute an interesting option and are the subject of intense research. For this, SPIONs, in conjunction with external magnetic fields, seem a suitable alternative for drug delivery by maintaining appropriate local drug concentrations at target site while reducing overall dosage and side effects.
<table>
<thead>
<tr>
<th>Brand name</th>
<th>PEG conjugates</th>
<th>Company</th>
<th>PEGylation</th>
<th>Indication</th>
<th>Approved Year</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adagen®</td>
<td>Pegademase bovine</td>
<td>Enzon</td>
<td>Random, multiple linear 5 kDa PEGs, amine PEGylation</td>
<td>Adenosine deaminase deficiency in patients with severe combined immunodeficiency disease who are not suitable candidates for, or who have failed, bone marrow transplantation</td>
<td>1990 (US)</td>
<td>(Hershfield, 1998)</td>
</tr>
<tr>
<td>Oncaspar®</td>
<td>Pegasparagase (PEG-L-asparaginase)</td>
<td>Enzon</td>
<td>Random, multiple linear 5 kDa PEGs, amine PEGylation</td>
<td>First-line acute lymphoblastic leukemia, acute lymphoblastic leukemia and hypersensitivity to asparaginase</td>
<td>1994 (US, EU)</td>
<td>(Ettinger, 1995)</td>
</tr>
<tr>
<td>Pegasys®</td>
<td>Peginterferon alfa-2a</td>
<td>Hoffmann –La Roche</td>
<td>Random, branched 40 kDa PEG with two 20 kDa linear PEGs, amine PEGylation</td>
<td>Alone or in combination with Copegus, indicated for the treatment of adults with chronic hepatitis C virus infection who have compensated liver disease and have not been previously treated with interferon-alfa, treatment of adult patients with HBeAg-positive and -negative chronic hepatitis B who have compensated liver disease and evidence of viral replication and liver inflammation</td>
<td>2002 (US, EU)</td>
<td>(Veronese and Pasut, 2005)</td>
</tr>
<tr>
<td>Product</td>
<td>Manufacturer</td>
<td>PEGylation</td>
<td>Indication</td>
<td>Approval Dates</td>
<td>Reference</td>
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<tr>
<td>PegIntron®</td>
<td>Peginterferon alfa-2b</td>
<td>Schering-Plough</td>
<td>Random, linear 12 kDa PEG, amine PEGylation</td>
<td>2000 (EU) 2001 (US)</td>
<td>(Veronese and Pasut, 2005)</td>
<td></td>
</tr>
<tr>
<td>Neulasta®</td>
<td>Pegfilgrastim</td>
<td>Amgen</td>
<td>Selective, linear 20 kDa PEG, N-terminal PEGylation</td>
<td>2002 (US) 2003 (EU)</td>
<td>(Kinstler et al., 2012)</td>
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</tr>
<tr>
<td>Somavert®</td>
<td>Pegvisomant</td>
<td>Pharmacia &amp; Upjohn</td>
<td>Random, 4 – 6 linear 5 kDa PEGs, amine PEGylation</td>
<td>2002 (EU) 2003 (US)</td>
<td>(Parkinson et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>Macugen®</td>
<td>Pegaptanib sodium</td>
<td>OSI/Pfizer</td>
<td>Selective, branched 40 kDa PEG with two 20 kDa linear PEGs, amine PEGylation</td>
<td>2004 (US) 2006 (EU)</td>
<td>(Kourlas and Schiller, 2006)</td>
<td></td>
</tr>
</tbody>
</table>
### Chapter 2

**Literature Review**

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer/Type</th>
<th>Sponsor</th>
<th>Use Case</th>
<th>Approval Year (US/EU)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mircera®</td>
<td>mPEG-epoetin beta; CERA</td>
<td>Roche</td>
<td>Anemia associated with chronic renal failure, including patients on dialysis and patients not on dialysis</td>
<td>2007 (US, EU)</td>
<td>(Topf, 2008)</td>
</tr>
<tr>
<td>Cimzia®</td>
<td>Certolizumab pegol</td>
<td>UCB</td>
<td>Reducing signs and symptoms of Crohn’s disease and maintaining clinical response in adult patients with moderately to severely active disease who have had an inadequate response to conventional therapy</td>
<td>2008 (US)</td>
<td>(Chapman et al., 1999)</td>
</tr>
</tbody>
</table>
2.7. METALLIC NANOPARTICLES: SUCCESSFUL ACTIVE TARGETING STRATEGY

In recent years, theranostic metallic nanoparticles (TMNPs) have shown potential application in field of magnetic resonance imaging (MRI) and colloidal mediators for cancer magnetic hyperthermia (Lu et al., 2007). Nanotechnology based imaging and therapy has been investigated independently and their understanding has now evolved to a point enabling the birth of theranostics agent. The term ‘theranostics’ was coined about a decade ago and was first used to describe diagnostic tests developed to guide personalized therapies. It may be defined as the combination of therapeutic and diagnostic agents on a single platform i.e. the development of theranostic nanoparticles (TNPs) that may simultaneously monitor and treat disease. Here diagnostic mean those agents which provide enhanced visibility of specific tissues by increasing the signal to noise ratio relative to surrounding tissues and provide a quick, high fidelity snapshot of the living system. Theranostic agent enables an entirely new category of clinical solution for oncological disorders, permitting early recognition of disease through the use of contrast agents combined with existing imaging modalities followed by tailored release of therapeutic agent. Advantage with the use of metallic nanoparticle based theranostic system in cancer therapeutics includes: (i) tumor targeting ligands, that bind to a particular tumor cell and are capable of sequestering anticancer drugs exclusively within tumor, thus reducing the accumulation of the drugs in healthy tissues, (ii) Large surface to volume ratio, that provides opportunity for surface modification with improved cell entry, (iii) Protection of the therapeutic agent from the biological milieu, (iv) Improved bioavailability of the anticancer agent (v) Additionally, MNPs can detect and attack the heterogeneous crowd of tumor cells, (vi) High drug loading capacity, (vii) Delaying the drug resistance and (viii) Increasing therapeutic index through oncological site specific delivery.

2.8. SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES (SPIONs): PRECISION TARGETED DRUG DELIVERY SYSTEMS

Evidence from ancient nanotechnology has put forward the concept of contemporary nanotechnology, which was first introduced by Dr. Feynman in 1979 at the American Physical Society meeting held at the California Institute of Technology, addressing the
advancement of knowledge in the scope and production of nano-materials of varying sizes, shapes, and/or compositions (Ferrari, 2005). Modified nanoparticles (NPs) between the molecular (10 nm) and bulk (200 nm) size range, with particular functional groups can overcome the drawbacks of traditional disease diagnostics and therapeutic agents, along with a number of advantages like low immunogenicity, improved treatment efficacy and fewer side effects. Unique material properties, like electronic properties, optical properties and magnetic properties, make NPs ideal candidates for surface engineering and functionalization, for delivering drugs to specific target sites in vivo. Large surface area-to-volume ratio of NPs facilitates relatively high loading of therapeutic agent, and provides platform for targeting molecules in a single domain. Targeting methods generally fall into one of two categories: (i) passive targeting, which relies on the physiological differences between cancerous and normal tissues; and (ii) active targeting, which either relies on conjugated ligand for a specific surface receptor on cancerous tissue, or on any external physical stimuli such as ultrasound, magnetic field, electric field, etc. Magnetic drug delivery offers potential for active targeting strategy due to some interesting properties it exhibits, such as superparamagnetism, high field irreversibility, high saturation field, extra anisotropy contributions or shifted loops after field cooling, relatively low cytotoxicity, long lasting contrast enhancement, improved delineation of tumor margins and low sensitivity to the surrounding water molecules.

2.8.1. Formulation development of superparamagnetic iron oxide nanoparticles (SPIONs)

Superparamagnetism is a phenomenon that occurs in magnetic materials which corresponds to the small size of a single magnetic domain which retains no remnant magnetization upon the removal of an external magnetic field. Biocompatibility and toxicity are the two major concerns of superparamagnetic formulation development for biomedical applications. Parameters determining biocompatibility and toxicity of SPIONs are: the size, nature of the magnetic core (for instance, magnetite, iron, nickel, cobalt, neodymium–iron–boron or samarium–cobalt) and the final hydrodynamic size including the outer coating shell (Mahmoudi et al., 2011). However, the use of magnetic materials such as cobalt and nickel are restricted due to their high toxicity and
susceptibility to oxidation. So far, SPIONs are the most commonly employed for biomedical applications. Colloidal SPIONs, such as magnetite (Fe₃O₄) or its oxidized form, maghemite (γ-Fe₂O₃), are remarkably biocompatible and extensively investigated as magnetic nanoparticles (MNPs) for biomedical applications.

Structure of a SPION consists of a core (usually, magnetite, Fe₃O₄, or maghemite, γ-Fe₂O₃) coated with a biocompatible polymer for stabilization and protection of the magnetic particle from its environment. These ferrite NPs have a cubic inverse spinel crystal structure with oxygen ions forming a close-packed cubic lattice and iron cations located at interstitial tetrahedral and octagonal sites. Magnetization arises from electron hopping between the Fe²⁺ and Fe³⁺ ions that coexist at the octahedral sites in Fe₃O₄.

Magnetic nanoparticles (MNPs) require a carrier liquid to make a uniform dispersion (known as ferrofluid) with a stabilizer (or repelling electric charge) to prevent them from aggregation. There are three prerequisites for MNPs to show the desired magnetic characteristics. First of all, MNPs must be sufficiently small and not undergo agglomeration and precipitation. Secondly, there should be no transition from a ferro- or ferrimagnetic state to an antiferromagnetic or diamagnetic state of MNPs. Thirdly, the particle material must have a high level of magnetizability. Most common magnetic fluids are usually composed of 3 to 15 nm-sized single domain magnetic particles coated with a molecular layer of stabilizer (surfactant, such as oleic acid), where the particles are suspended in a liquid carrier. The basic mechanism behind the stability of colloidal system is the thermal agitation of Brownian motion which keeps the particles suspended in liquid. During synthesis, it should be kept in mind that the particles must not to be too small, since at sizes less than 1 to 2 nm, their magnetic properties tend to disappear.

The magnetic force exerted on SPIONs by an external magnetic field gradient is the basis of magnetic targeting. Several physical parameters affect the effectiveness of therapy, such as the field strength, the gradient, and the volumetric and magnetic properties of the particles. SPIONs-mediated drug localization involves competition between forces exerted on the particles by the blood compartment of the body, and magnetic forces generated from the externally-applied magnetic field. Usually, a strong permanent magnet, such as Nd–Fe–B, fixed externally on the body over the target site, is responsible for generating a magnetic field gradient. The drug-magnetic carrier complexes, generally
in the form of a biocompatible ferrofluid, are injected into the patient via the circulatory system. The magnetic particles are retained at the target site by external, high-gradient magnetic fields when the applied magnetic forces exceed the linear blood flow rates in arteries (10 cm s\(^{-1}\)) or capillaries (0.05 cm s\(^{-1}\)), where they can deliver the therapeutic agent of interest (Mahmoudi et al., 2011).

2.8.1.1. Size vs stability of SPIONs

After the ferrofluid is synthesized, it needs to be prevented from sedimentation during the long-term storage period and for maintaining a homogeneous distribution.

According to Odenbach two aspects govern sedimentation of particles, viz. the density difference between the magnetic particles and the carrier liquid (energy in gravitational field) or the applied external magnetic field, which attracts the magnetic particles and accelerates them relative to the carrier liquid (Buschow, 2003).

If the thermal energy of the particles is able to keep them suspended, a stable suspension is achieved. Thermal energy can be expressed as:

\[
E_T = k_B T
\]

where, \( k_B \) is the Boltzmann’s constant, and \( T \) is the absolute temperature.

The thermal energy should be greater than their energy in the gravitational field or in a magnetic field gradient, respectively, for obtaining stable ferrofluid. The energy of the particles in the gravitational field is expressed by the following equation:

\[
E_g = \Delta \rho g V h
\]

where, \( \Delta \rho \) denotes the density difference between the particles and the carrier liquid, \( g \) is the acceleration due to gravity, \( h \) is a typical dimension of the sample, and \( V = \pi d^3/6 \) denotes volume of the magnetic particle with \( d \) being its diameter.

Comparing thermal (Equation 1) and gravitational energies (Equation 2) of the particles, we obtain an upper limit for the particle size as expressed below:

\[
d \leq \sqrt[3]{\frac{6k_B T}{\pi \Delta \rho gh}}
\]
Chapter 2

Assuming that magnetite particles are dispersed in water ($\Delta \rho \approx 4 \times 10^3 \text{ kgm}^{-3}$) and a typical height of the sample is $h = 0.1 \text{ m}$ ($g = 9.81 \text{ m/s}^2$, $T = 300 \text{ K}$), particles with a diameter less than approximately 12 nm are considered to be stable against sedimentation in the earth’s gravitational field.

Similarly, the stability against sedimentation in a magnetic field gradient can be checked. The stability against sedimentation implies that thermal energy needs to be large enough to permit the particles to move freely from the region with $H_0$ to the region with $H = 0$. In other words, thermal energy ($E_T$) needs to be greater than the energy of the particle in the field, $H_0$.

$$E_m = \mu_0 m H_0 = \mu_0 M_0 V H_0$$  \hspace{2cm} (4)

where, $m = M_0 V$ represents the magnetic moment of the particle, and $M_0$ denotes the spontaneous magnetization of the magnetic material ($M_0 = 4.5 \times 10^5 \text{ Am}^{-1}$ for magnetite, $\mu_0 = 1.2566 \times 10^{-6} \text{ VsA}^{-1}\text{m}^{-1}$ the permeability of free space). As seen from the above equation, the magnetic energy depends on the particle size. Thus, the stability criterion again leads to an upper limit for the particle size, as shown in the equation:

$$d \leq \sqrt[3]{\frac{6k_B T}{\pi \mu_0 M_0 H_0}}$$  \hspace{2cm} (5)

For magnetite particles and a typical step in field strength of $H_0 = 10^4 \text{ Am}^{-1}$, a maximum particle size would be about 10 nm. Thus, the particles need to have diameters in the order of 10 nm and less to satisfy the basic criterion for stability of a suspension of magnetite nanoparticles.

This implies a second group of stability criterion, since no agglomeration of the particles should take place. Agglomeration would lead to the growth of particles, and thus sedimentation, which would become efficient to destabilize the suspension. Magnetic particle–particle interaction or van der Waals forces of interaction are responsible for agglomeration processes in colloidal suspensions of magnetic particles. We can easily see that stability against magnetic agglomeration is achieved for magnetite particles with $d \leq$
10 nm, which means that a state in which the thermal energy of two particles is greater than their maximal magnetic interaction energy in contact:

\[ E_{dd} = \frac{\mu_0 \pi M_0^2 d^3}{72} \]  

(6)

leads one more to a size limit by:

\[ d \leq \sqrt[3]{\frac{144 k_B T}{\pi \mu_0 M_0^2}} \]  

(7)

The aspect of colloidal stability therefore provides the boundary condition for the production process that NPs with a diameter of about 10 nm have to be produced which must be covered with an appropriately chosen surfactant.

### 2.8.1.2. Synthesis of SPIONs

SPIONs, mainly magnetite (Fe\textsubscript{3}O\textsubscript{4}), maghemite (γ-Fe\textsubscript{2}O\textsubscript{3}) and hematite (α-Fe\textsubscript{2}O\textsubscript{3}) have been produced by a variety of synthetic processes to permit control over the particle size and shape. Proper tuning of both the size and coating of NPs is essential during synthesis of SPIONs to impart colloidal stability, and ease of ligand coupling for in vivo targeting. In general, synthesis procedures can be categorized into three approaches: chemical, physical and biological routes of synthesis (Table 2.3).

#### Table 2.3. Approaches for synthesis of SPIONs

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Physical</th>
<th>Biological</th>
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<tr>
<td>Co-precipitation</td>
<td>Gas-phase production</td>
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<td>Microemulsion</td>
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<tr>
<td>Thermal decomposition</td>
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</table>
2.8.1.2.1. Chemical routes of synthesis

Most SPIONs utilized or under investigation for in vivo application are predominately synthesized by chemical route of synthesis. In these processes, the control of the solution pH value and its environment is very critical for particle formation and its properties.

2.8.1.2.1.1. Co-precipitation method

Precipitation from solution is a fundamental method of crystallization. It involves nucleation and crystal growth as the principle pathways for solid formation. Herein, nuclei grow uniformly by diffusion from the solution to their surfaces. Ideally, uniform nucleation followed by crystal growth without further nucleation, leads to the synthesis of monodispersed NPs. However, multiple nucleations can also lead to uniform NPs by Oswald ripening. Herein, large uniform crystals are formed by crystal growth through the dissolution of small crystallites. Larger-sized uniform particles can also be obtained following aggregation of small crystallites through coalescence. Crystal growth in solution is interface-controlled up to a certain critical size, beyond which, the growth becomes diffusion controlled. Controlling the crystal growth step in the co-precipitation route is the key step to produce SPIONs.

A well-known co-precipitation method was first described by Massart in 1981, in which a 2:1 mixture of ferric (Fe$^{3+}$) to ferrous (Fe$^{2+}$) salts was precipitated through the addition of an acid or base in an oxygen-free environment to produce a black precipitate of spherical SPIONs of uniform sizes (Massart, 1981). Some researchers synthesized magnetite particles by co-precipitation from an aqueous Fe$^{3+}$/Fe$^{2+}$ solution in ratio of 3:2 using concentrated ammonium hydroxide in excess. An inert atmosphere (oxygen-free) during synthesis prevents the oxidation of magnetite to ferric hydroxide in the reaction medium. The chemical reaction taking place in the reaction mixture is depicted in the following equation:

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$$ \hfill (8)

Sugimoto and Matijevic established a co-precipitation method, where well-defined spherical SPIONs were prepared using ferrous salt (Fe$^{2+}$) in the presence of potassium nitrate and potassium hydroxide (Sugimoto and Matijević, 1980).
A comparison between Sugimoto's and Massart's methods for SPIONs synthesis was studied by Sen et al. (Sen et al., 2006). They found that Sugimoto's method produced larger particles (30 to 200 nm) of rhombic (stirred condition) and spherical (static condition) morphologies, whereas Massart's method produced smaller (<20 nm) spherical particles (Drmota et al., 2012).

A schematic representation of flow diagram for synthesis of ferrofluid by co-precipitation method is shown in Figure 2.9.

![Flow diagram for synthesis of ferrofluid by co-precipitation method.](image)

**Figure 2.9. Schematic diagram showing production of ferrofluid by co-precipitation method.**

2.8.1.2.1.2. Microemulsion method

A microemulsion (water in oil: w/o) can be defined as a thermodynamically-stable biphasic isotropic dispersion of two immiscible liquids, where water droplets act as nanoreactors in a continuous phase (oil) with the help of surfactant molecules. Microemulsion method is reported to be an alternative and more controlled method for the synthesis of SPIONs. In this method, iron precursors can be precipitated in aqueous
phase ("water pools"), specifically in the centre of the micelles and distributed homogenously in an oil phase. Iron oxides do not precipitate in organic phase as the iron precursors are unreactive in this phase. The surfactant molecules, responsible for micellization, provide a confinement effect that controls the particle size by limiting particle nucleation, growth and agglomeration.

By this method, mostly silica (SiO$_2$)-coated maghemite ($\gamma$-Fe$_2$O$_3$) NPs were synthesized _in-situ_ via the precipitation in two different microemulsion systems. Maghemite ($\gamma$-Fe$_2$O$_3$) NPs were prepared according to Schikorr’s reaction as depicted in the equation below:

$$\text{Fe}^{2+} + \text{Fe}^{3+} + 2\text{OH}^- + \text{O}_2 \rightarrow \gamma\text{-Fe}_2\text{O}_3 + \text{H}_2\text{O}$$ (9)

The surface silica coating of $\gamma$-Fe$_2$O$_3$ NPs prepared in microemulsion systems was functionalized with tetraethoxysilane (TEOS). An example of microemulsion consisting of anionic surfactant is water phase (aqueous solution of Fe$^{2+}$/Fe$^{3+}$ ions or aqueous solution of NH$_4$OH) in cyclohexane as the oil phase stabilized by sodium n-dodecyl sulfate (SDS) (Drmota et al., 2012). The second example of microemulsion system consisting of cationic surfactant is water phase (aqueous solution of Fe$^{2+}$/Fe$^{3+}$ ions or aqueous solution of NH$_4$OH) in 1-hexanol used as an oil phase where n-hexadecyl trimethyl ammonium bromide (CTAB) served as cationic surfactant (Drmota et al., 2012). In both the above cases 1-butanol was used as the co-surfactant.

SPIONs of size ranging from 200 to 400 nm were synthesized on a large scale by Zhang _et al_ (Zhang et al., 2008). But due to lower temperature of synthesis, preparation of adequate crystalline SPIONs suffered. On the other hand, highly crystalline iron oxide NPs were synthesized by Lee _et al_ at high temperature using an iron precursor of iron (III) acetyl acetonate (Lee et al., 2005). Though precipitation within reverse microemulsions has been successfully executed, its yield is very low and thus not suitable for biomedical applications. Vidal-Vidal _et al_ have reported the synthesis of oleylamine (or oleic acid)-coated maghemite NPs of narrow size distribution of 3.5±0.6 nm by the one-pot microemulsion method (Vidal-Vidal _et al_., 2006). Here, oleylamine acts as the precipitating and capping agent.
2.8.1.2.1.3. Hydrothermal method

The hydrothermal method is reported to be the oldest method, where magnetite particles are synthesized from iron precursors in aqueous medium at high-temperature (generally in the range from 130 to 250°C) and high vapor pressure (generally in the range of 0.3 to 4 MPa). Hydrothermal synthesis can be defined as a method of synthesis of single crystals that depends on the solubility of minerals in hot water under high pressure. This method produces SPIONs of uniform sizes as compared to co-precipitation and microemulsion techniques (where it is associated with polydispersity) and can easily be scaled up.

Several authors have reported the synthesis of iron oxide NPs by hydrothermal method. Wang et al have reported a one-step hydrothermal process to prepare highly crystalline Fe₃O₄ NPs of 40 nm without using the surfactants (Wang et al., 2003), whereas Zheng et al reported a hydrothermal route for preparing Fe₃O₄ NPs with diameter of 27 nm in the presence of a surfactant, sodium bis (2-ethylhexyl) sulfosuccinate (AOT) (Liu et al., 2002). Recently, Daou et al have reported that the SPIONs with an average size of 39 nm and good monodispersity have been synthesized by co-precipitation, followed by hydrothermal treatment at 250°C (Daou et al., 2006).

2.8.1.2.1.4. Sonochemical method

A recent development in the synthesis of SPIONs by sonochemical routes has been successfully explored. In this process, a high energy ultrasonication creates acoustic cavitations (formation, growth, and implosive collapse of bubbles in liquid) that generate a localized hotspot through adiabatic compression or shock wave formation within the gas phase of the collapsing bubble at temperature of about 5,000 K, pressures of 1,800 atm, and cooling rates in excess of 10¹⁰ K/s (Suslick, 1990). Monodisperse NPs of a variety of shapes can be synthesized by sonication of iron (II) acetate in water under an argon atmosphere; however, it lacks large scale synthesis. Vijayakumar et al synthesized 10 nm Fe₃O₄ powder by sonochemical synthetic route (Vijayakumar et al., 2000), whereas Pinkas et al developed a sonochemical synthetic method for preparing the amorphous nanoscopic iron oxide by sonolysis of Tris(acetylacetonato) iron(III) Fe(acac)₃ under argon with a small amount of added water (Pinkas et al., 2008).
2.8.1.2.1.5. Electrochemical deposition technique

Iron oxide thin films can be cathodically or anodically electrodeposited by a simple, manufacturable, inexpensive and fast technique for the synthesis of NPs, at nearly room temperature which can offer the versatility to control composition, crystallinity, and properties of the deposit by adjusting deposition conditions (Schrebler et al., 2006). Electrochemical Deposition under Oxidized Conditions (EDOC) has also been used to synthesize maghemite and magnetite NPs. In this method, the anode can be oxidized to metal ion species in solution and the metal ion is later reduced to metal by the cathode in the presence of stabilizers. This method, however, also lacks large-scale synthesis.

Zotti et al reported the cathodic electrodeposition of amorphous Fe₂O₃ thin films by reduction of Fe (III) perchlorate by dissolved oxygen in acetonitrile, which was later converted to maghemite thin films after heat treatment (Zotti et al., 1998). Schrebler et al electrodeposited amorphous and nanocrystalline γ-Fe₂O₃ thin films from electrolytes containing a mixture of FeCl₃ and H₂O₂ (Schrebler et al., 2006). Hydrogen peroxide reduced at cathode and caused an increase in local pH on the surface of the cathode favouring surface precipitation of Fe (OH)₃. Ferric hydroxides were later transformed to Fe₂O₃ by thermal annealing in air. Park et al synthesized crystalline maghemite NPs by cathodically electrodeposition at room temperature from FeCl₃ solution. Magnetic saturation (MS) of maghemite NPs was determined to be 66 emu g⁻¹ at 300 K (Park et al., 2008).

2.8.1.2.1.6. Thermal decomposition technique

Thermal decomposition of organometallic compounds offers good control over the SPIONs size, shape, and crystallinity as compared to other synthesis techniques. The reaction, however, occurs in organic solvent containing hydrophobic stabilizers and is a complicated reaction which requires relatively higher temperatures.

Synthesis of nanocrystalline γ-Fe₂O₃ from iron choline citrate, iron carbonate, and iron carboxylate has been successfully synthesized by thermal decomposition. Liu and Kim synthesized magnetic platelets from ethylene diamine by solvothermal route (Liu et al., 2007). Some researchers also reported solvent-free thermal decomposition route for the preparation of SPIONs (Maity et al., 2009). Although the thermal decomposition method has many advantages of producing highly monodispersed particles with a narrow size
distribution, it has a big disadvantage that the resulting NPs are generally only dissolved in non-polar solvents.

2.8.1.2.2. Physical methods of preparation of magnetic nanoparticles

2.8.1.2.2.1. Gas-phase production
In gas-phase reactors, morphology and other characteristics of NPs are determined by fluid mechanics and particle dynamics within a few milliseconds at the initial stages of the synthesis process. Particle production is governed by three major mechanisms:

(i) **Surface growth** - here NPs can be formed by a chemical reaction where precursor leads to the formation of product monomers by nucleation or direct inception.

(ii) **Coagulation** - it is an intrinsic mechanism where particles in a fluidized state move randomly due to Brownian motion and collide with each other along their trajectories. Due to strong adhesive forces or chemical bonds between the particles, coagulation of new particles takes place.

(iii) **Coalescence and fusion** - due to sintering processes in the high-temperature zones of the reactor, a reduction in the level of aggregation and formation of spherical particles takes place. Though it is an alternate process for NP production, due to very high temperatures of the reactor and an aggressive gaseous atmosphere, it restricts its use for the NP production in biomedical applications.

2.8.1.2.2.2. Electron beam lithography
High resolution electron beam lithography (HREBL) has already proved its efficiency for the production of NPs. The design of planar metallic nanoelectrodes can be made using a positive or negative resist for HREBL. But negative resists are associated with low resolution or swelling during development, possess a high degree of sensitivity to the process parameters, and require a long irradiation time. Mostly, poly-methylmethacrylate (PMMA) seems to be a good candidate for both positive and negative tones. Most authors have used PMMA as a positive resist. It has also been used as a negative resist successfully for the fabrication of quantum device structures. Benitez et al. reported self-assembly of iron oxide NPs by electron beam lithography with positive PMMA which was exposed to a focused 20-keV electron beam in a FEI QUANTA FEG 200 scanning electron microscope (SEM) to produce ≈ 220 nm thick layers (Benitez et al., 2010).
Ressier et al fabricated planar cobalt electrodes separated by a sub-10nm gap using HREBL with negative PMMA (Ressier et al., 2007).

2.8.1.2.2.3. Pulsed laser ablation

Though pulsed laser ablation was first developed in the 1960s, introduction of liquid phase pulsed laser ablation was first reported in 1987 by Patil and co-workers who produced iron oxides by using a pulsed laser to ablate a pure iron target in water (Patil et al., 1987). In this method, laser beam is focused through the liquid onto the surface of a solid target, immersed in a liquid medium. After hitting with laser, the surface of target metal vaporizes in the form of ablation plume. The species (atoms, ions or clusters) in the plume, travelling with high kinetic energy, collide and react with surrounding liquid molecules to extreme conditions (high temperature, high pressure, and high density due to intensity of the laser) and produce new compounds of original target and the liquid. This technique offers many distinct advantages. Firstly, it is a chemically ‘simple and clean’ synthesis, the final product being devoid of any byproducts and so, no need for further purification is there. Secondly, the experimental setup is of low cost and the parameters can easily be controlled. Lastly, the conditions are extremely confined, and high temperature and high pressure induced in the region favor the formation of unusual metastable phases. These advantages allow the combination of selected solid targets and liquid for fabrication of compound nanostructures with desired functions. Sasaki et al synthesized spherical NPs of a calcium–iron complex oxide of diameter ranging from 2 to 26 nm in diameter by pulsed laser ablation on silicon wafer substrates placed at off-axial positions against a target (Sasaki et al., 1998). Franzel et al synthesized SPIONs by laser ablation of Fe foil in ethanol which consisted of Fe₃O₄ and Fe₃C and showed a saturation magnetization of 124 emu/g (Franzel et al., 2012).

2.8.1.2.2.4. Laser-induced pyrolysis

SPIONs were obtained by laser-induced pyrolysis of iron pentacarbonyl vapours (FeCO₅) and ethylene (C₂H₂) vapours followed by controlled oxidation in a continuous flow reactor. The very high heating and cooling rates reached by the process >10⁵ °C/s due to the laser source cause nanosized particle formation. FeCO₅ is chosen due to its sufficiently high vapour pressure and low activation energy for breaking the metal–CO
bonds. By following this method, Bomatí et al synthesized SPIONs (maghemite, \( \gamma \)-Fe\(_2\)O\(_3\)) sized 10-30 nm showing well-constructed iron oxide core-shell structure (Bomatí-Miguel et al., 2006). They also investigated the dependence of magnetic properties on the particle size, iron oxide fraction, and temperature, whereas Martelli et al used sulfur hexafluoride (SF\(_6\)) as an energy transfer gas, preferred to C\(_2\)H\(_2\) to avoid ethylene fragmentation in N\(_2\)O presence, to form Fe\(_2\)O\(_3\) by the following equation (Martelli et al., 2000):

\[
2\text{Fe(CO)}_5 + 3\text{N}_2\text{O} \rightarrow \text{Fe}_2\text{O}_3 + \text{Residual Products}
\]

Here, N\(_2\)O was used as an oxygen donor to obtain more reactive atomic oxygen instead of O\(_2\) molecules and to favour iron oxidation. By this method, SPIONs with uniform and controllable particle size and monomodal size distributions in the range of 5 to 10 nm can be obtained in a single step which can be scaled to pilot plant dimensions.

2.8.1.2.2.1. High-energy ball milling

High-energy ball milling (HEBM) is an effective, low cost, high yield and simple technique for the production of metallic NPs. Due to high kinetic energy inside the ball mill, starting powder particles undergo repeated deformation, cold-work hardening, and fragmentation of premixed powders resulting in the formation of nanocrystalline powders. Due to the nature of HEBM, cold welding processes lead to an increase in the average particle size. Therefore, the best approach for the preparation of magnetic NPs is to use an appropriate surfactant and organic carrier liquid which enable us to obtain nanoscale particles. Chakka et al synthesized various metallic NPs of Fe, Co, FeCo, SmCo, and NdFeB with small size of around 30 nm by HEBM in the presence of surfactants (oleic acid, oleyl amine) and heptane as carrier liquid during the milling time of 1-50 h (Chakka et al., 2006). After a certain period of milling, steady-state equilibrium was attained when no further increase in size was achieved. After 90 h of milling \( \alpha \)-FeOOH (goethite) powder, superparamagnetic \( \alpha \)-Fe\(_2\)O\(_3\) (20 nm) NPs were synthesized by Wang et al through HEBM (Wang and Jiang, 2007).
2.8.1.2.3. Biological

Most of the chemical routes of synthesis for iron oxide NPs require toxic chemicals, harmful surfactants and non-polar organic solvents as carrier fluids. In addition, the physical methods are performed under harsh experimental conditions such as high temperature and pressure, thereby restricting their use in biomedical applications. Biological route of synthesis, on the other hand, offers an alternate, eco-friendly technique, regulated by variable experimental conditions of temperature, pH and pressure.

Biogenic processes used for the synthesis of iron oxide NPs are usually termed as biomineralization. Biologically-induced biomineralization (BIM) allows extracellular synthesis of magnetite crystals, where the metabolites of microbial cells (generally anaerobic bacteria) react with specific ions or compounds, either in solution or already adsorbed onto the cell surface, resulting in poorly crystalline mineral particle formation. Size distribution and crystal morphologies depend on the environmental parameters, like pH, pO\textsubscript{2}, pCO\textsubscript{2}, redox potential and temperature. Such types of biomimetic synthesis of SPIONs have been reported with Fe (III)-reducing bacteria (Shewanella sp., Geobacter sp., Thermoanaerobacter ethanolicus, etc.), sulphate-reducing bacteria (Archaeoglobus fulgidus, Desulfuromonas acetoxidans) and magnetotactic bacteria (Magnetospirillum magnetotacticum, M. gryphiswaldense). Fe (III)-reducing bacteria generally respire with oxidized Fe (III) compound in the form of Fe(III) oxyhydroxide under anaerobic conditions and secrete poorly crystalline Fe(II) into the extracellular environment. The Fe (II), so formed, then adsorbs onto excess ferric hydroxide grain and transforms into magnetite at high pH, as depicted in the equations below:

\[
\text{CH}_3\text{COO}^- + 8\text{Fe(OH)}_3 \rightarrow 8\text{Fe}^{2+} + 2\text{HCO}_3^- + 15\text{OH}^- + 5\text{H}_2\text{O} \quad (11)
\]
\[
2\text{OH}^- + \text{Fe}^{2+} + 2\text{Fe(OH)}_3 \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O} \quad (12)
\]

Some anaerobic sulfate-reducing bacteria (SRB) (Archaeoglobus fulgidus, Desulfuromonas acetoxidans) also produce magnetite. The SRB respire with sulfate anaerobically releasing H\textsubscript{2}S (dissimilatory sulfur reduction). The chemical reactions taking place are depicted below:
S + H⁺ + 4H₂ → HS⁻ + 4H₂O (13)

Fe (III) reduction by sulfide to yield Fe (II) and elemental sulfur:

2Fe(OH)₃ + HS⁻ + 5H⁺ → 2Fe²⁺ + S⁰ + 6H₂O (14)

Magnetite formation from Fe (III) and Fe (II):

2Fe(OH)₃ + Fe²⁺ → Fe₃O₄ + 2H⁺ + 2H₂O (15)

Magnetotactic bacteria (MTB) are gram-negative, heterogeneous group of aquatic microorganisms which contain single or multiple chains of magnetosomes. Upon magnetic interaction, magnetosomes orient along magnetic field lines due to the presence of intracellular membrane-bound crystals of magnetic iron mineral which consists of magnetite or greigite. This phenomenon is known as magnetotaxis. Magnetite formation in magnetotactic bacteria is a complex process involving numerous discrete steps. Magnetite formation inside magnetosome vesicles is first followed by uptake and transportation of reduced Fe (III), i.e. Fe (II) inside the vesicle, and then magnetite formation seems to be the controlled biomineralization of magnetite.

Gudadhe et al reported the synthesis of magnetic iron oxide NPs by fungal cell filtrate Phoma glomerata (plant pathogen) (Gudadhe et al., 2011). Nanoparticle Tracking and Analysis (NTA) by LM-20 revealed the polydispersed NPs with an average size of 56 nm, which was further confirmed by transmission electron microscopy (TEM).

Some authors have also reported green synthetic strategy by utilising non-toxic chemicals, environmentally benign solvents, and renewable materials for the production of SPIONs. Senthil and Ramesh used the plant extract of Tridax procumbens leaves for the green synthesis of iron oxide NPs (Senthil and Ramesh, 2012). The plant extract possesses biomolecules such as carbohydrates, proteins and lipids, which can be used as reducing agents to react with ferric ions, resulting into the formation of iron oxide NPs.

2.8.1.3. Surface modification of SPIONs

Apart from superparamagnetic nature and a high degree of saturation magnetization of SPIONs, small size, combined with colloidal stability are vital parameters for their applications in drug delivery. Small sizes are required to allow transport through the vascular system or biodistribution of particles (Vidal-Vidal et al., 2006) whereas colloidal stability is associated with charge and surface chemistry of NPs, which give rise
to both steric and columbic repulsions. In general, nanometer size particles tend to sediment due to the attractive Vander Waals forces which minimizes the total surface or interfacial energy resulting into low surface area and larger sizes. Hence, the stabilization of SPIONs by surface modification either during or after the synthesis process is an important issue in the context of drug delivery.

Surfactants, such as oleic acid, lauric acid, alkane sulphonic acids, and alkane phosphonic acids, due to their amphiphilic in nature, have been used as stabilizers since a long period (Sahoo et al., 2001). Most of the surfactant-mediated syntheses used organic solvents, i.e. hexadecane, toluene, n-hexane, etc., as carrier fluid which restricts its biomedical application.

In addition, polymer coating can also keep the chemical moiety unchanged and prevent it from biodegradation when exposed to the biological system. Also, therapeutic active ingredient can be loaded into the polymer either by covalent attachment, adsorption or entrapment in matrix. Various natural polymers, like starch (Kim et al., 2003; Wang and Zhang, 2007), dextran (Bautista et al., 2005), gelatin (Gaihre et al., 2008), chitosan (Kim et al., 2005), albumin (Milenyi et al., 1990), ethyl cellulose (Ma et al., 2004), and phospholipids (Meincke et al., 2007), are reported which can provide nontoxic, biocompatible carriers for both drug delivery and as a contrasting agent. Similarly, synthetic polymers, viz. poly(ethylene glycol) (PEG) (Mondini et al., 2008), polyvinyl alcohol (PVA) (Chastellain et al., 2004), polylactic acid (PLA) (Chen et al., 2008a), alginate (Ma et al., 2008), poly methyl methacrylate (PMMA) (Singh et al., 2005), polyacrylic acid (PAA) (Arbab et al., 2003), polyvinylpyrrolidone (PVP) (Lee et al., 2008), polylactic-co-glycolic acid (PLGA) (Schleich et al., 2013), poly(glycerol monoacrylate) (Wan et al., 2006), poly(glycerol monomethyl acrylate) and tri-block copolymers (Harris et al., 2003), provide bio functionality and resistance to physiological conditions such as pH and enzymes. Coating with amorphous silica on SPIONs was first reported by Philipse et al which are known to be biocompatible but not biodegradable (Philipse et al., 1994). Due to hydrophilic nature, the silica-coated particles were well dispersed in aqueous suspensions.

The advantages of surface modification are: (i) sufficient loading of therapeutics with high dose effectiveness, (ii) controlled release of therapeutics in target the environment,
(iii) reduction/elimination of adverse effects of cytotoxic agents, and (iv) enhanced ability to avoid uptake by the reticuloendothelial system (RES). During designing of stealthy NPs, it has been reported that RES interaction of SPIONs < 40 nm in diameter tends to be influenced more by surface modification than their size. Negatively charged surfaces facilitate uptake and clearance via the RES as compared to positively charged surfaces due to more interaction with plasma proteins. Generally, hydrophilic and neutral surfaces can possess long circulation properties by escaping their uptake by the RES. Surface modification of SPIONs can be confirmed by Fourier Transform Infrared measurements (FT-IR). The iron oxide core itself shows a characteristic vibration at 598 cm$^{-1}$, related to the Fe–O bonds. Native iron oxide cores exhibit the characteristic bands of the asymmetric stretching, symmetric stretching, and scissoring of CH$_2$ at 2,919, 2,850; and 1,436 cm$^{-1}$, respectively.

2.8.1.4. Characterizations of SPIONs

The particle size is an important parameter affecting colloidal stability, superparamagnetic properties, and achieving enhanced permeability and retention (EPR) effect. Ideally, particle size should be between 10 nm to 100 nm to remain in the circulation for a long time after injection such particles can evade RES. In suspended formulations, particle size is determined by dynamic light scattering (DLS), whereas transmission electron microscope (TEM), Scherrer’s analysis of X-ray diffractograms (XRD) and extended X-ray absorption fine structure (EXAFS) determine the crystallite size in its dry state. Dynamic light scattering, the way in which light scatters off particles in suspension, has the potential to yield a great deal of information on the mean sizes based on intensity, number and volume distribution of particles (Figure 2.10).

TEM is the most powerful technique to determine the crystallite and particle sizes and their morphology. This technique gives information on the size distribution, as well as details of the core–shell structure due to the difference in electron density of core and coating materials. The TEM image of the SPIONs shows spherically-shaped, monodispersed particles with a size of around 10 nm, as reported by most authors. Apart from the information about the crystallographic structure, chemical composition, and
physical properties of the materials obtained by XRD diffractogram, one can determine
the size of NP by Scherrer analysis of XRD by the following equation:

\[ \tau = (K\lambda)(\beta\cos\theta)^{-1} \]  

(16)

where, \( \tau \) is the mean size of the ordered (crystalline) domains, \( K \) is the shape constant
(usually 0.89, but it varies with the actual shape of the crystallite), \( \lambda \) is the wavelength of
the X-ray used, \( \beta \) is the width of the peak at half height in radians and \( \theta \) is the Bragg
angle.

![Image](image.png)

Figure 2.10. Size distribution curves by intensity (A, B); size distribution by
number (C); and size distribution by volume (D).

In general, the colloidal stability of nanoparticles dispersed in aqueous media can be
expressed by the zeta potential. It can be qualitatively described as the nature and
behaviour of the surface groups in solution at a certain pH in the presence of an
electrolyte. Quantitatively, it can be measured as the potential difference between the
slipping plane in the electronic double layer and the bulk potential. Although colloidal
stability is governed by the electrostatic and steric repulsion, zeta potential measurements
usually give a good indication about stability. A high zeta potential (absolute value higher
than 25–30 mV) value is an indication of the dispersion stability of SPIONs due to the electrostatic interaction (Philipse et al., 1994).

By following Bragg’s equation, XRD patterns of the native iron oxide particles revealed diffraction peaks at 110, 220, 311, 400, 422 and 511, which are the characteristic peaks of the Fe$_3$O$_4$ crystal with a cubic spinel structure with the respective hkl indices from the Joint Committee on Powder Diffraction Standards (JCPDS). It has been found that after coating of the surface of SPIONs, there is no influence on the crystalinity of the magnetite. From XRD studies, it has been found that magnetite has an inverse spinel structure with oxygen forming a face-centred cubic (FCC) closely packed arrangement and Fe cations occupying the interstitial tetrahedral and octahedral sites. The structure of maghemite is similar to that of magnetite except that all Fe ions are in a trivalent state (Fe$^{3+}$).

In the case the magnetic particle, each of the particle is carrying a net magnetic moment (m) and the whole liquid will have a paramagnetic character and can thus be described using Langevin’s equation for the magnetization of a paramagnetic system.

$$M = M_s \left( \text{ctgh} \alpha - \frac{1}{\alpha} \right) = M_s L(\alpha) \quad (17)$$

where, $M_s = \phi M_0$ denotes the saturation magnetization of the ferrofluid given by the product of the volume concentration $\phi$ of suspended magnetic material and its spontaneous magnetization ($M_0 = 4.5 \times 10^5$ A/m for magnetite). The character L denotes the Langevin function, with $L(x) = \text{ctgh} \, x - 1/x$. 

For normal magnetite ferrofluids, the magnetization curve does not show any hysteresis as is expected for a pure paramagnetic system. The measure of the magnetization curve provides fundamentally important data about the composition of the fluid. First of all, the saturation magnetization provides a direct measure of the volume concentration $\phi$ of the suspended magnetic material in the fluid. The saturation magnetization is usually obtained from the measured magnetization curve by plotting the magnetization versus $1/H$ as approximated by following equation:

$$M = M_s \left( 1 - \frac{1}{\alpha} \right) = M_s \left( 1 - \frac{kT}{\mu_0 m H} \frac{1}{H} \right) \quad (18)$$
Chapter 2

Literature Review

The increasing and decreasing magnetization curves were almost mirror images. The hysteresis loop had negligible coercivity at room temperature. The saturation magnetization was found to be 45.90 emu/g. With this saturation magnetization, the SPIONs can be easily and quickly separated from a suspension by an adscititious magnetic field. Because of superparamagnetism, there will be no magnetic interactions among SPIONs in a zero adscititious magnetic field environment, which explains the easy dispersion of the particles in solution.

2.8.2. Applications of SPIONs

2.8.2.1. As therapeutics

SPIONs can be used as therapeutic purposes by delivering either drugs or proteins and peptides or DNA and genes to the target sites as illustrated below in Figure 2.11.

![Figure 2.11. Therapeutic applications of SPIONs.](image)

2.8.2.1.1. Drug delivery

SPIONs can be used for targeted drug delivery to the desired site with the aid of an external magnetic field. Additionally, by integrating SPIONs with targeting ligands or
surface modifications with different coating materials provide the opportunity to increase the bioavailability of loaded therapeutic drug within the diseased tissue. SPIONs, modified with o-carboxymethyl chitosan (OCMCS) and folic acid (FA), provide combined strategy to improve their biocompatibility and tumor specificity in Folate-receptor positive tumors in vivo. These tailored SPIONs can evade the reticuloendothelial system and improve plasma pharmacokinetics and their internalization into cells. PEGylated SPIONs loaded with anticancer drug can be conjugated with tumor-targeting ligands on the distal ends of the polyethylene glycol (PEG) arms. Yang et al. investigated the synthesis and release characteristics of poly(ethyl-2-cyanoacrylate) (PECA)-coated SPIONs loaded with hydrophobic cisplatin and hydrophilic gemcitabine (Yang et al., 2006). He found that cisplatin was shown to exhibit a sustained release in comparison to the more rapid release of gemcitabine. Alexiou et al. investigated on starch (provides biological stabilization and sites for chemoabsorptive/electrostatic binding to MTX)-coated SPIONs, loaded with mitoxantrone into VX2-squamous cell carcinomas on the hind limbs of rabbits, which completely suppress tumors within 35 days of treatment (Alexiou et al., 2006).

SPIONs have been evaluated as drug carries for a variety of chemotherapeutic agents to treat diseases ranging from rheumatoid arthritis to various tumors. Mostly, different anticancer drugs are reported, which can be targeted to the specific site with the help of these SPIONs. Mykhaylyk et al. studied the pharmacokinetics of doxorubicin (DOX)-loaded magnetic nanoconjugate in mice after i.v. injection in a non-uniform stationary magnetic field of 210 mT and gradient of 200 mT/cm (Mykhaylyk et al., 2005). This magnetic field was effective in increasing the DOX-nanoparticle bioavailability at the target site. Electron spin resonance (ESR) analysis demonstrated that the DOX-nanoparticles substantially decreased DOX bioavailability in the heart and kidney, reduced hepatic clearance, resulting into increased plasma bioavailability, compared to free DOX. Quan et al. reported tumor targeting capability of DOX and SPIONs loaded into human serum albumin (HSA) matrices by MRI and immunostaining (Quan et al., 2011). The 50 nm SPIONs caused the translocation of DOX across cell membranes followed by nuclear accumulation in vivo. Its anticancer activity was found superior to that of Doxil® and free DOX in 4T1 breast cancer model. DOX was also investigated
with modified SPIONs to target lung carcinoma and magnetic lipid NPs for intracellular delivery. Kohler et al demonstrated a sustained release of methotrexate (MTX) in breast and brain tumor cells where MTX was covalently conjugated to amine-functionalized NPs through amide bonds (provide stability to the drug under i.v. conditions) (Kohler et al., 2005). MTX was released from the SPIONs over a range of pH values and in the presence of lysozymes which cleaved amide bond between drug and amino terminal. Early clinical trials of SPIONs loaded with epirubicin provided successful accumulation in the target site in about half of the patients included in the study (Lübbe et al., 2001). Dilnawaz et al fabricated dual drug-loaded, coated SPIONs for targeted cancer therapy, where paclitaxel and rapamycin were evaluated for their combined anti-proliferative activity against MCF-7 cell lines (Dilnawaz et al., 2010). The combination drug-loaded GMO-coated SPIONs showed lower IC₅₀ value as compared to native drug and GMO, depicting the synergistic effect of different drugs towards cancer treatment which can minimize their biotoxicity in normal cells.

2.8.2.1.2. Protein and peptide delivery
The human epidermal-growth-factor receptor-2 (HER2), responsible for the control of cell proliferation, migration, and differentiation is over expressed on the surface of tumor tissues and metastatic deposits but not in normal tissues. Trastuzumab (Herceptin™), a monoclonal antibody (mAb) has been developed with high affinity and specificity for HER2. HER2, overexpressed in 20–30% of breast cancers, can be used as the tumor targeting marker for the treatment of metastatic breast cancer. Herceptin™-conjugated SPIONs as mAb-targeting agent were also investigated as targeted carriers for tumor treatment. After the conjugation of HER2 antibody with GMO- SPIONs, the cellular uptake of GMO-SPIONs by MCF-7 cells was increased by 3 times (Dilnawaz et al., 2010). The magnetite immunoliposomes loaded with anti-HER2 showed antiproliferative effects on SKBr3 breast cancer cells in vitro and can be used in combination for both antibody therapy with hyperthermia (Ito et al., 2004). Conjugation of mAb to SPIONs can be a useful method for the detection of tumor cells, especially by MRI technique. Presence of Herceptin-nanoparticle conjugates on SKBR-3 and T47D human breast carcinoma cell lines surface was confirmed by Prussian blue iron staining method. Detection of breast cancer cells using targeted SPIONs and ultra-sensitive magnetic field
sensors in breast cancer cell lines was visualized by confocal microscopy, Prussian blue histochemistry, and magnetic relaxometry. Chlorotoxin (CTX), a peptide originally purified from the venom of the *Leiurus quinquestriatus* scorpion, has high affinity for a variety of tumors of neuroectodermal origin, particularly brain tumors such as gliomas. Recently, CTX has been conjugated with SPIONs to target brain tumor cells (Veiseh et al., 2010b).

2.8.2.1.3. DNA and genes delivery (Magnetofection)

In order to fabricate SPIONs as effective carriers for DNA and gene delivery, the surface of the particles must first be functionalized with cleavable linkers to attach the target molecules. Adeno-associated virus (AAV) encoding green fluorescent protein (GFP) was conjugated to magnetic microspheres using a cleavable heparin sulfate linker which resulted in enhanced transduction efficiency in both C12s cells cultured *in vitro* and *in vivo* following intramuscular injection to 129/svJ mice (Mah et al., 2002). One can also employ electrostatic interactions between the negatively-charged phosphate backbone of DNA and positively-charged particle surface alternative for the attachment of DNA. Positively-charged polymer Polyethyleneimine (PEI), due to the presence of a large number of secondary amine groups throughout its chain, can be used for the attachment of DNA (Abdallah et al., 1996). PEI-coated magnetic particles have been also reported which were covalently coupled to the surface of composite iron oxide, dextran silica particles using glutardialdehyde linkers demonstrated the association of DNA vectors with SPIONs which enhanced the transfection efficiency by reducing the duration of gene delivery to as little as 10 min. Due to the short half-life *in vivo*, lack of specificity, and poor diffusion across cell membranes, the delivery of genes and their resulting transfection efficiencies are often limited, which can be overcome by SPIONs loaded with antisense oligodioxynucleotides (ODNs) or gene vectors. Dendrimer-modified SPIONs loaded with antisense survivin ODNs were also developed to target breast and liver cancer cells. SPIONs can also be used as carriers for the delivery of small interfering RNA (siRNA). A research study was done on SPIONs labeled with a NIFR dye for imaging *in vivo*. NIFR dye was covalently bound with siRNA, loaded onto SPIONs which was shown to silence green fluorescent protein (GFP) production in a GFP expressing xenograft tumor mouse model.
2.8.2.2. As diagnostics

SPIONs have been examined widely as MRI contrast agents for the detection, diagnosis, and anatomical information of solid tumors at its earliest stages, in some cases, even prior to disease manifestation. SPIONs are better candidates for imaging purposes as compared to gadolinium-based contrast agents due to their slower renal clearance and high relaxation values. RES-mediated uptake of SPIONs, due to their small particle size, can be used for clinical imaging of liver tumors. With a diameter of 5–10 mm, SPIONs can be effective in identification of lymph node metastases under MRI. Currently, SPIONs are used clinically in improving the delineation of brain tumor boundaries and quantifying tumor volumes.

Chelation of carboxylic acid groups of poly(ethylene glycol)-poly(aspartic acid) block copolymer with Fe on the surface of SPIONs exhibited enhanced pancreatic cancer imaging in xenografts of the human BxPC3 cell line in nude mice model (Kumagai et al., 2009). For hepatic imaging, $^{99}$Tc-adsorbed composite particles were used which showed marked biodistribution in the left hepatic lobe of pigs (Cao et al., 2008). Optically-active SPIONs have been synthesized by linking NPs to fluorescent dyes or by coating with other inorganic agents (for example, gold, quantum dots) can be used clinically in oncology due to surface plasmon resonance.

Now-a-days, development of metal-doped iron oxides is an upcoming field in molecular imaging due to the enhancement in magnetic properties. Recently, Lee et al reported the synthesis and characterization of various spinel metal ferrites with a composition of $\text{MFe}_2\text{O}_4$, where M is a +2 cation of Mn, Fe, Co or N (Lee et al., 2007a). This group demonstrated that MnFe$_2$O$_4$ NPs were non-toxic as compared to other composites in vitro and possessed higher magnetic susceptibility suggesting their use as an ultrasensitive MR imaging probe. Investigators like Baldi et al examined the synthesis and coating of CoFe$_2$O$_4$ SPIONs for use as magnetic nanocarriers (Baldi et al., 2007), whereas Rana et al investigated nanocrystalline NiFe$_2$O$_4$ as drug carriers (Rana et al., 2007).
2.9. OBJECTIVE

➢ To design and develop nanocarriers with targeting ability for selective localization and prolonged retention at the desired site to increase the bioavailability of the loaded drug for the effective management of solid tumors.

2.10. RATIONALE OF STUDY

➢ To formulate hemo-compatible Cremophor® EL free formulations of paclitaxel (PAC) as potential nanocarriers to target solid tumor with enhanced i.v. bioavailability.

➢ To improve the therapeutic efficacy of PAC by co-administering with curcumin (CUR), a potent chemopreventive molecule.

➢ To formulate and evaluate therapeutic efficacy of PAC and CUR-loaded stealth PEGylated lipidic nanocapsules (D-LNCs) for i.v. delivery in Ehrlich Ascites tumor bearing mice model.

➢ To formulate PAC and CUR-loaded superparamagnetic iron oxide nanoparticles (D-SPIONs) for the effective management of solid tumor by magnetic drug targeting delivery in Ehrlich Ascites tumor bearing mice model.