INTRODUCTION

CANCER

Cancer is a class of diseases characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis). This unregulated growth is caused by a series of acquired or inherited mutations to DNA within cells, damaging genetic information that define the cell functions and removing normal control of cell division. These invasive tissues are said to be malignant. The word tumor (‘swelling’ in Latin) refers to any mass of abnormal tissue, but may be either malignant (cancerous) or benign (non-cancerous).

1.1.1 FACTS ABOUT CANCER

Cancer is a leading cause of death worldwide. From a total of 58 million deaths worldwide in 2005, cancer accounts for 7.6 million (or 13%) of all deaths. The main types of cancer leading to overall cancer mortality are:

- Lung (1.3 million deaths/year);
- Stomach (almost 1 million deaths/year);
- Liver (662,000 deaths/year);
- Colon (655,000 deaths/year) and
- Breast (502,000 deaths/year).

More than 70% of all cancer deaths in 2005 occurred in low and middle-income countries. Deaths from cancer in the world are projected to continue rising, with an estimated 9 million people dying from cancer in 2015 and 11.4 million dying in 2030. The most frequent cancer types worldwide are:

- Among men (in order of number of global deaths): lung, stomach, liver, colorectal, oesophagus and prostate,
- Among women (in order of number of global deaths): breast, lung, stomach, colorectal and cervical.
1.1.2 QUICK CANCER FACTS

- 40% of cancer can be prevented (by a healthy diet, physical activity as well as by banning tobacco).
- Tobacco use is the single largest preventable cause of cancer in the world.
- One-fifth of cancers worldwide are due to chronic infections, mainly from hepatitis B viruses HBV (causing liver), human papilloma viruses HPV (causing cervix), Helicobacter pylori (causing stomach), schistosomes (causing bladder), the liver fluke (bile duct) and human immunodeficiency virus HIV (causing Kaposi sarcoma and lymphomas).

1.1.3 STAGES OF TUMOR DEVELOPMENT

Various Stages of tumor development can be explained as follows:

- Tumor evolution commences when a cell (or some of likes) within a normal population sustains genetic mutation that expands its tendency to proliferate when it would normally rest.
- Genetically altered cell and its offspring continue to appear normal, but they reproduce excessively and lead to a condition termed as hyperplasia. After some time (months or years) one in a million of these cells sustain additional mutation with subsequent loss of control on cell growth.
- The offspring of this cell not only proliferate excessively but also appear abnormal in shape and in orientation. The tissue is now said to exhibit a condition referred as dysplasia. After sometime, a further mutation that alters cell behavior results.
- The influenced and genetically altered cells become more abnormal in growth and appearance. If the tumor mass do not invade through any boundaries between tissues, it is termed as in situ tumor. This tumor may stay contained indefinitely or some cells may acquire additional mutations.
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- A malignant tumor results if the genetic changes allow the tumor mass to initiate invasion of underlying tissue mass to initiate invasion of underlying tissue and to cast off cells into the blood or lymph. The defector cells may install new tumor loci (metastases) throughout the body.

![Diagram of tumor development](image)

Fig. 1.1: Tumor development from initial carcinogenesis to diffusion limited maximal size (Peppas and Blanchette, 2004).

The tumor microenvironment plays a critical role in malignant tumor progression and treatment. One of the few consistently different characteristics of solid tumor tissue compared with normal tissue is the existence in the former of significant areas which are either transiently or chronically hypoxia. Hypoxic cells represent a therapeutic challenge in that these cells are refractory to radiation therapy and resistant to many of the cytotoxic drugs used in chemotherapy.
1.1.4 CANCER TREATMENTS

The treatments may be divided into different categories based on their goal and mode of action. Often the different types of treatment are used in combination, either simultaneously or sequentially. The following section describes some of the most common forms of cancer treatment and they are arranged in the order in which they often occur. The actual types of treatment used and the order in which they are used are decisions made by the physician and patient.

The types of treatment and their objective are described briefly below:

- **Surgery**: Is the first line treatment for many solid tumors. In cases, the cancer is detected at an early stage. The surgery may be sufficient to cure the patient by removing all cancerous cells. Benign growths may also be removed by surgery.

- **Radiation**: It is may be used in conjunction with surgery or chemotherapy. The goal of radiation is to kill the cancer cells directly by damaging them with high energy beams.

- **Chemotherapy**: A term used for a wide array of drugs used to kill cancer cells. Where drugs acts by damaging the dividing cancer cells and preventing their further reproduction.

- **Hormonal Treatments**: Prevent cancer cell growth by preventing the cells from receiving signals necessary for their continued growth and division.

- **Specific Inhibitors** *(Stephen et al., 2000)*: These classes of drugs are relatively new in the treatment of cancer. They act by targeting specific proteins and processes that are limited primarily to cancer cells or that are much more prevalent in cancer cells. Inhibition of these processes prevents cancer cell growth and division.

- **Antibodies**: This treatment involves the use of antibodies to target cancer cells. The antibodies used in the treatment of cancer have been manufactured for use as drugs.

- **Biological Response Modifiers**: These treatments involve the use of naturally occurring normal proteins to stimulate the body’s own defenses against cancer.
• **Vaccines**: The purposes of cancer vaccines are to stimulate the body's defenses against cancer. Vaccines usually contain proteins found on or produced by cancer cells. By administering these proteins, the treatment aims to increase the response of the body against the cancer cells.

### 1.1.5 STRATEGIES TO OVERCOME DIFFICULTIES IN CANCER TREATMENT

The main problems currently associated with systemic drug administration are: even biodistribution of pharmaceuticals throughout the body; lack of drug specific affinity toward a pathological site; the necessity of a large total dose of drug to achieve high local concentration; non-specific toxicity and other adverse side-effects due to high drug doses. Because of these drawbacks of systemic drug administration and the toxicity of anti-cancer drugs to both tumor and normal cells, the efficacy of cancerous chemotherapy is often limited by serious side-effect. A strategy could be to associate anti-cancer drugs with colloidal nanoparticles, with the aim to increase selectivity of drugs towards cancer cells while reducing their toxicity towards normal tissues ([Brigger et al., 2002](#)). The accumulation of intravenously injected nanoparticles onto tumor cells relies on a passive diffusion or convection across the leaky and hyper permeable tumor vasculature ([Yuan, 1998](#)), which was called passive targeting. Indeed, the uptake of nanoparticles can also result from active targeting, a specific recognition in the case of ligand decorated nanoparticles ([Moghimi et al., 2001](#)). To date, many distinctive active targeting nanoparticles, such as folate-conjugated albumin nanoparticles ([Zhang et al., 2004](#)), monoclonal antibody conjugated nanoparticles ([Sheng et al., 1995](#)), magnetic albumin nanoparticles ([Gong et al., 2004](#)), pH and temperature-responsive polymeric micelles ([Kwon et al., 1995; Chung et al., 2000; Jeong et al., 2003](#)), have been investigated in drug delivery applications.
1.2 SOLID TUMOR

The word tumor does not always imply cancer. Some tumors (collections of abnormally growing cells) are benign (not cancerous). In discussing tumors that are malignant (cancerous), however, the term solid tumor is used to distinguish between a localized mass of tissue and leukemia. (Leukemia is actually a type of tumor that takes on the fluid properties of the organ it affects—the blood.)

Solid tumors are constituted by variable amounts of neoplastic and stromal cells, the latter comprising fibroblasts, blood/lymphatic vessels and immune-competent cells, mainly macrophages and lymphocytes. A wide array of biologically active molecules are available in this milieu, either in soluble form or associated to proteins of the extracellular matrix. These include, for instance, growth factors for tumor cells and for the newly formed blood vessels, chemo attractants for immune cells recruited into the tumor mass, and a vast number of proteolytic enzymes which actively remodel the surrounding matrix.

1.2.1 SOLID TUMOR TARGETING

Targeting tumors by means of their vascular endothelium is a promising strategy which utilizes targets that are easily accessible and endothelial cells that are genetically stable and do not develop resistance against therapeutic agents (Molema et al., 1997; Schnitzer, 1998). The vascular endothelium in solid tumors differs from that of normal tissues with respect to their anatomy and the expression of functional receptors on the cell surface. The existing vasculature in all solid tumors (more than 1-2 mm in diameter) gives rise to new blood vessels in order for providing the increasing demands for nutrients and oxygen by the rapidly proliferating tumor cells. This process is known as angiogenesis, and is marked by activation of existing endothelial cells, which show an elevated expression of cell adhesion molecules and proteolytic enzymes. (Folkman et al., 1989).

Thus, the vascular endothelium provides many targets for cancer therapy, including the endothelial cells, and specific stromal components which are highly accessible to any
system present in circulation and thus, can be used to target drugs/drug-carriers (Molema et al., 1997).

Tumor targeting of a cytotoxic agent refers to the passive accumulation of nano-scaled drug carriers to solid tumors, followed by active internalization into tumor cells (Minko et al., 2004). The internalization of drug, either alone or along with a carrier, is required for cell death because most cytotoxic drugs act intracellularly (Kim, 2005 and, Wardwell and Massion, 2005). Important considerations of nano-sized carriers for improved passive tumor targeting include the surface property, shape and size for a given tumor. Surface PEGylation of the carriers is regarded as the gold standard for longer residence time in the blood and improved biocompatibility (Crawford, 2002). Spherical nanocarriers with diameters from 40 to 300 nm are typically used for passive targeting (Demeneix et al., 2004). Active targeting carriers have either monoclonal antibodies (mAb) (Richter and Zhang, 2005), binding fragments (Spiridon et al., 2004) specific to a tumor associated surface antigen or a ligand binding to its corresponding receptor on the tumor cell surface. It has been clearly established that active targeting results in higher accumulation of carriers in tumors (Normanno et al., 2005) albeit mAb or ligand conjugated to carriers may not guarantee long-range interactions with tumor cells. The term ‘active targeting’ is a misnomer as the carriers do not actively seek their target, in this case the tumor areas, but exert specific interactions with tumor cells only upon contact. Most therapeutic systems rely on the receptor-mediated endocytotic pathway for internalization into cells. This pathway leads to the entrapment and, to a large extent, degradation of transported biomolecules in lysosomes. The use of cell penetrating peptides, like the HIV peptide TAT, has the advantage of avoiding this pathway and taking the cargo directly into the cell. TAT-mediated cytoplasmic uptake of drug conjugated polymers (Kopecek et al., 2003 and, Hyndman et al., 2004), plasmid DNA (Hyndman et al., 2004), bacteriophages (Paschke and Hohne, 2005), magnetic nanoparticles of about 10–20 nm in diameter (Nitin et al., 2004) and even liposomes having a diameter of 200 nm (Fretz et al., 2005) has been documented in the literature (Wang et al., 1999, Riedel et al., 2000, Troy et al., 1994).

Different kinds of solid tumors are named for the type of cells of which they are composed:
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- **Sarcomas**
  Cancers arising from connective or supporting tissues such as bone or muscle.

- **Carcinomas**
  Cancers arising from the body's glandular cells and epithelial cells, which line body tissues.

- **Lymphomas**
  Cancers of the lymphoid organs such as the lymph nodes, spleen, and thymus, which produce and store infection-fighting cells. These cells also occur in almost all tissues of the body, and lymphomas therefore may develop in a wide variety of organs.

1.3 MECHANISM OF LIGAND ENTERNALIZATION

Receptor mediated endocytosis (RME) allows for a more rapid means of ligand targeted internalization compared to that of untargeted complexes. Depending on the receptor-dependent or independent endocytic path, the intracellular trafficking path can also be controlled. For example, those macromolecules taken up by clathrin-dependent RME are typically destined for lysosomal degradation; whereas, clathrin-independent RME internalization leads to endosomal accumulation and sorting to a nondegradative path. Appropriate selection of targeting agents could therefore allow for controlled delivery to the lysosomes or endosomes to alleviate conditions associated with these individual organelles, including cancer (Castino et al., 2003), Alzheimer's disease (Tate and Mathews, 2006), and most importantly lysosomal storage diseases (LSDs).

Targeted molecules have to follow following order for endosytosis:

1.3.1. Clathrin-dependent endocytosis

Clathrin-mediated endocytosis serves as the main mechanism of internalization for macromolecules and plasma membrane constituents for most cell types. Clathrin-dependent mechanisms that have been identified thus far include the well characterized RME pathway, including cell adhesion molecule (CAM) assisted RME. In these
processes, intracellular vesicles form invaginations in the membrane that are coated by the triskelion protein clathrin populating the cytoplasmic face of the membrane. Coated pits cover 1–2% of the plasma membrane surface area and allow for rapid intracellular vesicle budding, occurring as quickly as 1 min. Clathrin aids in vesicular formation from multiple sites, including endosomes and TGN.

### 1.3.1.1. Receptor-mediated endocytosis.

Clathrin-dependent RME is the most investigated vesicular pathway for targeted drug delivery. In addition to a well characterized internalization scheme, numerous receptors, including transferrin, asialoglycoprotein (Lu and Low, 2002), epidermal growth factor (EGF) (Biessen et al., 1999), and chemokine (Mamot et al., 2003), serve as high affinity binding sites and have been investigated for their use in targeting to different cell types. Ligands bind specific cell surface receptors, signaling their directional movement towards clathrin underlined pits in the membrane. Receptor–ligand complexes accumulate in pits through the aid of multisubunit complexes, or adaptins, which bind to a four amino acid signaling sequence in the carboxy terminus of the receptor. Here, receptors are concentrated in the coated pits prior to invagination, minimizing the amount of extracellular fluid which can be retained in the vesicle. After binding and internalization, ligands and/or receptors may be recycled or ultimately enzymatically degraded in the lysosomal compartment depending on cellular requirements.

### 1.3.2 Trafficking mechanisms for clathrin-independent endosomal transport

Endocytic internalization is a highly coordinated and active process allowing whereby a cell internalizes an area equivalent to its entire plasma membrane every hour (Biragyn et al., 2004). The cellular mechanics associated with endocytic internalization allows for several different forms of vesicle formation including nonspecific adsorptive endocytosis and clathrin-dependent (CME) and independent RME. All approaches involve the invagination of lipid rich membrane regions which lead to intracellular release of membrane bound vesicles housing extracellular fluid and substances including
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Nutrients (Renau-Piqueras et al., 1985), macromolecules (Huang and Swaan, 2000), and receptors. Vesicle acidification is a hallmark feature of CME, which may lead to the inadvertent hydrolysis of pH-sensitive macromolecules. On the other hand, clathrin-independent internalization enables the intracellular accumulation of materials along a less destructive path as compared to CME. The two major pathways include caveolae-mediated endocytosis (CvME) and the less characterized lipid raft internalization. Both methods share a variety of common features making it difficult to form a common distinction (Lamaze et al., 2001); however, both may serve as targets for a less destructive form of targeted drug delivery.

1.3.2.1 Caveolin assisted receptor mediated endocytosis

Although clathrin-dependent endocytosis is the predominant endocytosis pathway in most cells, alternative, parallel uptake pathways have been recently identified, such as CvME. Caveolae are flask-shaped invaginations, ranging from 50 to 100 nm in diameter, making up more than 10% of the plasma membrane for endothelial cells. Caveolar morphology differs dramatically between cells types making it complicated to definitively infer on common structural features. These invaginations are held in place by underlying actin filaments in the cytoskeleton, where certain membrane proteins are found to concentrate. Unlike clathrin mediated endocytosis, caveolae-assisted endocytosis is a triggered process that involves subsequent complex signaling. Molecular based approaches investigating caveolae-mediated endocytosis have aided in the recognition of membrane proteins associated with this version of cellular entry. The most prevalent protein associated with caveolae structures is caveolin-1, which is pertinent to caveolae formation in the membrane and subsequent vesicular production as well as stabilization from a sub-membrane raft-dependent endocytic process. Antibodies directed at clathrin-associated endocytic components, such as endosomes, lysosomes, and trans golgi network (TGN), do not participate in the post-internalization trafficking of these endosomes. From this point, contents are delivered to subcellular (non-lysosomal) compartments (Pelkmans et al., 2001). For example, for SV40, the vesicular contents are transported to the endoplasmic reticulum where the virus remains until it is transported to
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the nucleus (Pelkmans and Helenius, 2002). This pathway could be utilized for targeted macromolecular delivery into cells through the binding of caveolae-associated membrane receptors. Ligands known to be internalized through receptor-dependent caveolae-mediated endocytosis include folic acid (Chang et al., 1992), albumin, and cholesterol. Folic acid, or vitamin B₉, appears an especially attractive target for targeted drug delivery. For example, folate targeted poly(ethylene glycol) (PEG)-coated nanoparticles are found to bind to folate receptors allowing for caveolae-assisted endocytosis, followed by the formation of intracellular vesicles which can be visualized by confocal microscopy (Dauty et al., 2002). In addition, albumin, which interacts with endothelium by binding to albondin (gp60) receptor, is used in vascular targeting through its caveola mediated uptake mechanism (Schnitzer, 2001). Caveolae-directed systems have been used to target chemotherapeutics to a nondegradative pathway, where pH sensitive bonds have allowed for drug release followed by diffusion across the endosomal membrane directed to the nucleus (Murthy et al., 2003). Characterizing the proteins and receptors pertinent to caveolar transport will aid in the design of targeted drug carriers which bypass the harsh environment of the lysosomes to render this trafficking pathway less harmful to drug complexes (Bathori et al., 2004).
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Fig 1.2; Representative fate of clathrin mediated RME of asialoglycoprotein (ASGP) and its receptor (ASGPr) upon endocytosis. After binding of ASGP to its receptor, the receptor–ligand complex is internalized in a clathrin-coated pit that pinches off to become a coated vesicle. The clathrin coat then depolymerizes to triskelions, resulting in an early endosome. This endosome fuses with a sorting vesicle (late endosome). Lowering of pH causes ASGP dissociation from ASGPr. A receptor-rich region buds off to form a separate vesicle that recycles multiple ASGPr back to the plasma membrane. ASGP-containing vesicles ultimately fuse with lysosomes, wherein it is degraded to amino acids and sugar (Dalton et al., 2003).

1.4 Diagnosis of Cancer

Various methods used to diagnose the cancerous cells are:

- **Endoscopy** (examination of an interior organ with a viewing instrument that has flexible glass or plastic fibers that transmit light)

- **Pap test** (removal of a small sample of cells from the cervix to be examined under a microscope)

- **Blood tests**
• **Biopsy** (removal of small amounts of suspect tissue for examination under a microscope)

• **Imaging techniques** such as X-rays, CT scans (X rays that give a three-dimensional image), magnetic resonance imaging (MRI), and ultrasound imaging.

### 1.5 Present Approaches for Cancer Therapy

Knowledge of the mechanisms of carcinogenesis in human epithelial tissues results in the development of strategies for disrupting this process and thereby preventing cancer. Following types of basic approaches are used to combat cancer:

• Inhibition of the growth of tumor cells by cytotoxic bioactives, for example, interference in DNA synthesis or structure or in the protein involved in cell division.

• **Antiangiogenesis therapy**, i.e. inhibition in the formation of the blood vessels that supply the tumor.

• **Hormonal therapy**, i.e. inhibition of the growth of hormone-dependent tumors by blocking the action of prostatic and breast cancer, respectively.

• **Target specific therapy**, i.e. specific interference in the molecular mechanism of cell growth and programmed cell death that is out of control in case of cancer.

• **Antimetastatic therapy**, i.e. inhibition or prevention of invasive growth and metastasis spread.

• **Immunostimulant therapy**, i.e. reinforcement or restoration of the functions of the immune system.

### 1.6 Limitation of Conventional Cancer Therapy

Great progress has been made in the treatment of selected malignancies and approximately 50% of all malignancies can be cured by current treatment strategies. Non-surgical methods of cancer treatment, primarily radiation therapy and chemotherapy, conventionally earlier used for cancer therapy.

The majority of these cures are achieved by surgery that is if the disease has not spread throughout the whole body. Radiotherapy and chemotherapy used alone or in combination have greatly improved the management of patients with a variety of solid
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and hematological malignancies. Chemotherapy has curative potentials in patients with various hematological malignancies, testicular cancer and germ cell tumors. Despite improvements in the treatment of most metastatic solid tumors, these remain largely incurable. Reasons for this are insufficient tumor selectivity of anti-cancer agents and poor penetration within the tumor mass (Jain, 1996). Another problem after surgical removal of the solid tumor is that metastatic cells that are resistant to conventional chemotherapy often remain. The currently used chemotherapeutic agents are the drugs with narrowest therapeutic indices in all medicines. Thus, their nonselective toxic effects on normal tissues restrict the dose of anticancer agents.

Selective delivery of cytotoxic drugs into malignant tumors may overcome these limitations. Even highly toxic agents could be rendered safer and more effective, if it were possible to direct them only into the tumor because high drug concentrations within the tumor could be obtained while sparing the normal tissues.

1.7 Tumor Targeting Via Novel Drug Delivery System

Chemotherapy has become an integral component of cancer treatment for most cancers. Despite the last 30 years of effort on oncology drug discovery, conventional chemotherapeutic agents still exhibit poor specificity in reaching tumor tissue and are often restricted by dose limiting toxicity. The combination of developing controlled release technology and targeted drug delivery may provide a more efficient and less harmful solution to overcome the limitations found in conventional chemotherapy. Recent interest has been focused on developing nanoscale delivery vehicles capable of controlling the release of chemotherapeutic agents directly inside cancer cells. Cancer cell targeting by NDDS aims at increasing selectivity and overcoming biological barriers, while transporting higher drug amounts to the tumor site.

Generally, targeting comprises of following strategies:

- Unique tissue physiology of the target (passive targeting).
- Specific recognition of target cells by carrier-conjugated molecules (active targeting).
- Localized external energy activation.
- Synergistic combination of the above strategies.

In tumor targeting all the above-mentioned strategies are exploited.
1.7.1 Passive versus Active Drug Targeting

In drug targeting, two types of strategies can be distinguished: passive targeting and active targeting. In the case of passive targeting, the carrier–drug complex is often delivered to macrophages and other cells of the monocyte-phagocytic system (MPS). This leads to gradual degradation of the carrier and (slow) release of the liberated drug from the cells either into the blood circulation or into the tissue environment. By size exclusion, extravasation of the carrier–drug complex can be limited; thereby preventing the drug from being distributed to non-target sites. As a consequence, toxicity can be reduced. Active targeting should lead to a higher therapeutic concentration of the drug at the site of action. This can be accomplished by cell specific delivery of the drug. In the ideal case, the dose of the drug can be reduced and side effects can be diminished. The majority, if not all, active drug targeting strategies exploit receptor-based drug targeting principles, in which receptor-specific ligands attached to the carrier–drug complex or directly to the drug itself deliver the drug to the target cell of choice. Depending on the subsequent routing of the receptor complex, the drug will arrive in a specific compartment in the target cell.

Targeting ligands explored for cancer therapy include antibodies and antibody fragments (Dinauer, 2005), vitamins (Na, 2003), peptides (Fahr, et al., 2002), folate (Leamon and Reddy, 2004) and transferrin (Vyas and Sihorkar, 2000). The choice of appropriate ligand is based on its specificity, stability, availability and selective display of its corresponding pair on the target cells, as well as its cost. In addition to the above considerations, conjugation chemistry (Nobs, 2004), density and accessibility of the ligand (Torchilin, 2001), need to be properly designed for efficient vector targeting.

1.7.2 Molecular Targets for Tumor Therapy

The molecular targets on the surface membrane of malignant cells may conveniently be divided into following categories and could be targeted using the counter ligand or specially designed antibodies (Vyas et al., 2001):

- Altered expression of cell adhesion molecules and their ligands.
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- Altered expression of certain receptors otherwise expressed by all eukaryotic cells, like insulin receptors and MHC class-I associated compound receptors.
- Exquisite expression of receptors during certain stages of cellular differentiation, like transferring receptor (TfR), ferritin receptor, folate receptors, apo-lipoprotein receptor, c-kit receptors, and MHC class-II associated receptors.
- Altered expression of certain growth factors (epidermal growth factor receptor) and certain angiogenic peptides.
- Expression of surface determinants on malignant cells, like Ia antigens and tumor associated antigens (TAA).
- Expression of tumor vasculature epitopes, either of the endothelial cells or of the basement membrane supporting the endothelial cells or tumor stroma components.

Table 1.1: Various receptors over-expressed by tumor cells*

<table>
<thead>
<tr>
<th>Target Receptors</th>
<th>Expression</th>
<th>Delivery strategies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate</td>
<td>Ovarian carcinomas and epithelial tumors</td>
<td>Folic acid-Cys₂⁸⁷ (Pseudomonas exotoxin) conjugate, Folic acid-maytansinoid (DM1) conjugate</td>
</tr>
<tr>
<td>Fucose</td>
<td>Leukaemia</td>
<td>N-(2-hydroxypropyl) methacrylamide co-polymer conjugates bearing N-linked fucosylamine and daunomycin</td>
</tr>
<tr>
<td>Transferrin</td>
<td>T-cell leukaemia, haematopoietic/tumor cells</td>
<td>Tf-methotrexate, Tf-adriamycin conjugate; Tf-polycation (poly-L-lysine/protamine) conjugate, Biotinylated Tf-DNA conjugate</td>
</tr>
<tr>
<td>Lipoprotein</td>
<td>Certain tumors</td>
<td>Porphyrin loaded lipoprotein (Photodynamic therapy)</td>
</tr>
<tr>
<td><strong>Down-regulation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asialoglycoprotein (ASGP)</td>
<td>Cirrhotic or tumor bearing livers</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>Certain tumors</td>
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</tbody>
</table>

*Adopted from Vyas et al., 2001
1.8 Carrier System

Carrier is one of the most important entities required for successful transportation of loaded drugs. The choice of carrier system to be used in drug targeting strategies depends on which target cells should be reached and what drug needs to be delivered. Carriers can be divided into soluble and cellular carriers. Polymeric carriers bearing physically entrapped or chemically conjugated drugs are an attractive strategy for improving the efficiency of tumor targeting. Polymer-conjugated drugs and nanoparticulate show prolonged circulation in the blood and accumulate passively in tumors even in the absence of targeting ligands (Parka et al., 2008).

1.9 Nanoparticles (NPs)

The colloidal carriers based on biodegradable and biocompatible polymeric system have largely influenced the controlled and targeted drug delivery concepts. NPs are sub-nanosized colloidal structures composed of synthetic or semi synthetic polymers.

Polymeric NPs defined as solid particle with a size in the range of 10-1000nm allow encapsulation of drug inside the polymeric matrix (Cascon et al., 2002). Because of their size, the NPs can also be coupled with targeting ligands to provide site-specific delivery, which is use full in cancer therapy.

1.9.1 Advantages of NPs as Drug Carriers

1. High stability (i.e. long shelf life).
2. High carrier capacity (i.e. many drug molecules can be incorporated in the particle matrix).
3. Feasibility of incorporation of both hydrophilic and hydrophobic substances.
4. Feasibility of variable routes of administration, including oral administration and inhalation.
5. They control and sustain release of the drug during the transportation and at the site of localization, altering organ distribution of the drug and subsequent clearance of the drug so as to achieve increase in drug therapeutic efficacy and reduction in side effects.
6. Their particle size and surface characteristics can be manipulated to achieve both passive and active drug targeting after potential administration.

7. They can be used as long acting depot preparation.

8. During transit in vivo they prevent the degradation and retard the release of the drug by minimizing the interaction of loaded drug with blood component.

9. Drug loading can be relatively high.

10. Without involving any chemical reaction the drug can be incorporated into the system, which is the possession to protect the drug.

11. Site-specific targeting can be achieved by attaching targeting ligands to surface of particles or use of magnetic guidance.

12. The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular etc.

In spite of these advantages, NPs do have limitations. For example, their small size and large surface area can lead to particle-particle aggregation, making physical handling of NPs difficult in liquid and dry forms. In addition, small particles size and large surface area readily result in limited drug loading and burst release. These practical problems have to be overcome before NPs can be used clinically or made commercially available.

While compared to liposomes the NPs possess the advantage of offering better protection to drug against chemical degradation there is no or little leakage problem. Depending on the nature of drug and polymer, a higher payload might be achieved.

1.9.2 Preparation of NPs

NPs can be prepared from a variety of matrix materials such as proteins, polysaccharides and polymers (synthetics and naturals). The selection of matrix materials depends on many factors like;

1) Size of NPs required.

2) Inherent properties of the drugs for examples aqueous solubility and stability.

3) Surface characteristics of NPs such as permeability and charge.
4) Degree of biocompatibility, toxicity and biodegradability.
5) Drug release profile desired.
6) The antigenicity of final product.

1.9.3. Method of Preparation

NPs have been prepared most frequency by three methods: (1) amphiphilic macromolecule cross-linking.; (2) polymerization of monomers; and (3) precipitation of preformed polymers.

1.9.3.1 Amphiphilic Macromolecule Cross-linking

NPs can be prepared from amphiphilic macromolecules, proteins and polysaccharides. The technique of their preparation involves firstly, the aggregation of amphiphile(s) followed by further stabilization either heat denaturation or chemical cross-linking. These processes may be occurring in a biphasic O/W or W/O type dispersion systems, which subdivide the amphiphile(s) prior to aggregative stabilization (Vyas and Khar, 2002).

1.9.3.2 Polymerization of Monomers

Several methods have been used for prepared biodegradable NPs from polymers, polylactide (PLA), polyglycolide (PLG), poly (D, L-lactide-co-glycolide) (PLGA) and poly (cyanoacrylate) (PCA) by polymerization of monomers based method. Two different approaches are generally adopted for the preparation of NPs using in situ polymerization technique.

- Methods in which the monomers to be polymerized is emulsified in a non- solvent phase (emulsion polymerization).
- Methods in which the monomers are dissolved in a solvent that is non-solvent for the resulting polymer (dispersion polymerization).

In emulsion polymerization methods, the monomers are dissolved in an internal phase while in the case of dispersion polymerization, it is taken in the dispersed phase. It is interesting that in either of the case, following polymerization, the polymer tends to be
insoluble in the internal phase or disperse phase thus result in to an ordered suspension of nanospheres.

**1.9.3.3 Precipitation of Preformed Polymers**

In these methods, the hydrophobic polymer and hydrophobic drugs is dissolved in a particular organic solvent followed by its dispersion in a continuous aqueous phase, in which the polymer in soluble.

**1.9.3.3.1 Solvent Evaporation Methods**

The most common method used for the preparation of solid, polymeric NPs is the emulsification–solvent evaporation technique. The preparation of particles by the classical method follows the general protocol of dissolving the polymer in a water immiscible, volatile organic solvent, which is then emulsified with an aqueous phase to stabilize the system. The organic solvent is then evaporated inducing the formation of polymer particles from the organic phase droplets. The solvent evaporation method was described by (Niwa et al., 1995) and has since been widely used to prepare particles from a range of polymeric materials, particularly PLA and PLGA. This technique has been successful for encapsulating hydrophobic drugs. However, results for incorporation of hydrophilic bioactive agents have been poor. A modification of this procedure has led to the protocol favored for encapsulating hydrophilic compounds and proteins viz., the double or multiple emulsion technique. First, a hydrophilic drug and a stabilizer are dissolved in water. The primary emulsion is prepared by dispersing the aqueous phase into an organic solvent containing a dissolved polymer. This is then re emulsified in an outer aqueous phase also containing stabilizer (Barrera et al., 1993). From here, the procedure for obtaining the NPs is similar to the single emulsion technique for solvent removal. The main problem with trying to encapsulate a hydrophilic molecule like a protein or peptide-drug is the rapid diffusion of the molecule into the outer aqueous phase during the emulsification.
General procedure of a solvent displacement process used for NP preparation

The main drawback of this method, besides the problem of preparing particles, which are sub 200nm in diameter, is the need for the removal of excipients postproduction. Any residual organic solvents will have toxicological implications. In addition the excess surfactant used is difficult to remove. Another limitation is that surfactant must be present for preparation of NPs in order to stabilize the system. Particles therefore cannot be produced naked and then post adsorbed with a surfactant (Ruan et al., 2003).

Scheme: 1, Method of Nanoparticle preparation.
1.9.3.3.2 Solvent Diffusion or Spontaneous Evaporation Method

This is the modified version of solvent evaporation method in which the water-soluble solvent along with water insoluble organic solvent is used as an oil phase. Due to spontaneous diffusion an interfacial turbulence is created between the two phases leading to the formation of small particles. A considerable decrease in particle size is achieved as the concentration of water-soluble solvent increase.

1.9.3.3.3 Supercritical Fluid Expansion Method

Conventional methods like solvent evaporation, concervation and polymerization often required the use of toxic solvents or surfactants. Recently the field of supercritical fluids has been investigated as an approach to the preparation of sub micron sized particles with safer encapsulation (Dong and Feng, 2004). The rapid expansion of supercritical solutions consists in saturating a supercritical fluid with the substrate(s), then depressurizing this solution through a heated nozzle into a low pressure chamber in order to cause an extremely rapid nucleation of the substrate(s) in form of very small particles or fibers, or films when the jet is directed against a surface that are collected from the gaseous stream. The major merits of these processes include: production of organic solvent free particles, mild operating temperatures for processing biological materials, and easier micro-encapsulation of drugs for controlled release of the therapeutic agents. Unfortunately, none of these techniques can produce small protein particles in the sub-micron range less than 300 nm (Shekunov et al., 2006).

1.9.3.3.4 Interfacial Polymer Deposition (IPD) Method

The IDP method was first described by (Fessi et al., 1995). The technique involves addition of polymer, dissolved in a water miscible solvent (usually acetone) into an aqueous non-solvent under stirring. The non-solvent is usually an aqueous surfactant or drug solution without surfactant. The rapid diffusion of solvent into the aqueous phase causes a decrease in the interfacial tension between the two phases which, together with the increased interfacial surface area created by the turbulence, results in the formation of small droplets of organic solvent without the need for high shear mechanical stirring. The solvent then diffuses further into the aqueous phase and water concurrently diffuses into the solvent droplets, resulting in the formation of polymer particles from the droplets. Particles are stabilized by a layer of polymer deposited at the interface. Thus, polymer
Introduction

properties may alter the physicochemical properties at the interface as explained in the Marangoni effect (Carla et al., 2000).

Decreased miscibility of organic solvents with water is associated with an increase in their resultant interfacial tension and thus increases the size of the particles. The higher the viscosity of the organic phase, greater the surface tension and particles size. An increase in molecular weight of polymers is associated with a decrease in the number of end carboxyl groups and hence lowers the zeta potential of the resulting particles. Additives present in the formulation may also significantly affect this surface charge. The presence of surfactant in the system acts as a stabilizer to prevent coalescence of the droplets. Surfactants like PVA were important to form NPs in a technique like emulsification as here they prevent coalescence of newly formed droplets. A distinct advantage of the IPD method is that there are no residual solvents left in the system, which is important from a toxicological point of view. Another advantage, from the viewpoint of using particles as a drug carrier, is that the particles produced are in the nanometer size range.

1.9.3.3.5 Spray Drying

In the classical spray drying technique the polymer and drug are dissolved in an organic solvent and sprayed through a fine nozzle. Solid, spherical particles form on the immediate evaporation of the solvent. High temperatures are generally employed in this process, which can create problems, particularly in the encapsulation of peptides, and proteins that are easily denatured. Spray drying produces particles that are in the micrometer size range and hence will not be considered further here.

1.9.3.3.6 Salting Out

Bindschaedler and co workers patented this technique in 1988 (Bindschaedler et al., 1988). The technique involves the preparation of particles by an emulsification technique but avoids the use of chlorinated solvents. In brief, a saturated salt solution containing a stabilizing agent such as PVA is added under stirring to an acetone solution of the polymer. An o/w emulsion forms as the salt prevents the water and acetone mixing. Sufficient water is then added to allow the acetone to diffuse into the external aqueous phase and induce particle formation. From the perspective of drug encapsulation, this
method is most appropriate for water insoluble compounds, although the loading of water-soluble compounds can be improved by techniques such as altering the pH of the aqueous phase. Salts permeate biological systems and are crucial for life. However, salts also affect the stability of proteins. It has been reported since many years that neutral salts perturb various protein structures in ways that go well beyond simple, nonspecific charge effects (Dominy et al., 2002).

1.9.3.3.7 Complex Coacervation

Complex coacervation is a phase separation process that spontaneously occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution. Compared to other methods, this process can be performed entirely in an aqueous solution and at low temperature and thus has a better chance to preserve activity of the encapsulated substances. The colloidal particles produced are in the nanometer or micrometer scale depending on the substrates or the processing parameters used for example; pH, ionic strength and polyelectrolyte concentrations (Kaibara et al., 2000). The major drawback of this technique is that complex coacervates have low drug loading efficiency and poor stability. Therefore, cross-linking of the complex by chemical reagents such as toxic glutaraldehyde is necessary.

1.10 PEGYLATION

PEGylation, defined as the covalent attachment of poly (ethylene glycol) (PEG) chains to bioactive substances. The technique has become the leading approach for overcoming most of the aforementioned limits of biologics and the number of PEGylated products on the market is continuously increasing, see Table 1.2, together with the number of new conjugates entering clinical trials (Maggon, 2007; Bailon and Won, 2009; Ryan et al., 2008; Pasut and Veronese, 2009; Pasut and Sergi, 2008). In particular, PEG reduces the rapid kidney clearance of a given protein by increasing its hydrodynamic volume, prevents the immunogenicity by acting at different levels as shown in Fig. 1.3, reduces protein aggregation owing to a repulsion between PEGylated surfaces (see next section) (Basu et al., 2006; Hinds et al., 2000) and increases thermal stability of proteins (Dhalluin et al., 2005). Many studies on this technology report that the conjugated proteins have unchanged secondary and tertiary structures (Digilio et al., 2003; Hinds and
Introduction

Kim, 2002; Meng et al., 2008; Veronese et al., 2007). Nevertheless, a reduction in biological potency is usually common after PEGylation due to the steric entanglement of polymer chains during protein/receptor recognition process: research is particularly active in addressing this issue and recent proposals are summarized and discussed in this review. PEGylation has also been investigated as DDS for low molecular weight drugs but in this field, despite some interesting results, it has not reached the same success of protein PEGylation. Nevertheless, the development of PEG-small drug conjugates, especially those bearing a targeting residue or a combination of two drugs, is an intense area of research. Consequently, this review will also examine some proposals in this area.

Table 1.2 PEGylated products in clinical practice (adopted from (Santucci et al., 2006)).

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Indication</th>
<th>Approval year</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-adenosin Deaminase (Adagen®)</td>
<td>Several combined immunodeficiency disease (SCID)</td>
<td>1990</td>
<td>Levy et al., 1988</td>
</tr>
<tr>
<td>PEG-asparaginase (Oncaspar®)</td>
<td>Leukemia</td>
<td>1994</td>
<td>Reddy et al., 2002</td>
</tr>
<tr>
<td>PEG-interferon α2b (Pegintron®)</td>
<td>Hepatitis C</td>
<td>2000</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>PEG-interferon α2a (Pegasys®)</td>
<td>Hepatitis C</td>
<td>2001</td>
<td>Reddy et al., 2002</td>
</tr>
<tr>
<td>PEG-human growth mutein Antagonist (Somavert®)</td>
<td>Acromegaly</td>
<td>2002</td>
<td>Trainer et al., 2000</td>
</tr>
<tr>
<td>PEG-G-CSF (Neulasta®)</td>
<td>Nettropenia</td>
<td>2002</td>
<td>Kinstler et al., 2002</td>
</tr>
<tr>
<td>PEG-ant-VEGF aptamer (Pegaptanib, Macugen™)</td>
<td>Wet age related muscular degeneration</td>
<td>2004</td>
<td>Ng et al., 2006</td>
</tr>
<tr>
<td>PEG-erytropoitin (Mircera®)</td>
<td>Anemia associated With chronic kidney Disease</td>
<td>2007</td>
<td>Macdougall, 2005</td>
</tr>
<tr>
<td>PEG-anti-TNF Fab’ (Cimzia®)</td>
<td>Rheumatoid arthritis Crohn’s disease Chronic gout</td>
<td>2008</td>
<td>Wong, 1991</td>
</tr>
<tr>
<td>PEG-uricase (Pegloticase, Krystexxa)</td>
<td></td>
<td>2010</td>
<td>Sherman et al., 2008</td>
</tr>
</tbody>
</table>
1.10.1. Basic features of PEG and PEGylation

Methoxy PEG, CH$_2$O–(CH$_2$CH$_2$O)$_n$–H, is at the basis of all approved PEGylated products either in its linear or branched form. Two key properties of this polymer are its great flexibility, owing to the absence of bulky substituents along the chain, and the high hydration of the polymeric backbone (Harris and Chess, 2003). PEG is quite unique in its behavior with water because its good solubility markedly contrasts with the hydrophobicity of similar polymers like poly (methylene oxide) and poly (propylene oxide), which differ by only one methylene unit less or more, respectively (Israelachvili, 1997). Furthermore, PEG is also soluble in many organic solvents and can form thin monolayers at the air–water interface, a typical property of amphiphilic molecules. Some properties of the polymer can vary significantly with molecular weight and, to a lesser extent, with concentration. Surfaces, modified with high molecular weight PEGs, will repel each other (Klein and Lukham, 1986; Lukham and Klein, 1986; Kuhl et al., 1994); conversely, if PEG is free in solution and repelled from specific surfaces it promotes an attraction between these surfaces, as shown with cells and liposomes (Boni et al., 1981; Boni et al., 1984; Rupert 1988; kuhl et al., 1996), accordingly with the theories of inert polymer mediated forces. The last behavior is not a common rule because PEG can also be attracted to the surface of certain vesicles, cells or macromolecules, and therefore induces surfaces repulsion or attraction on the basis of PEG molecular weight, concentration and temperature. Nevertheless, under normal circumstances, low molecular weight PEG promotes attraction (cells/vesicles aggregation) while high molecular weight PEG induces repulsion (stabilization of dispersed cells/vesicles) (Israelachvili, 1997; Boni et al., 1981; Boni et al., 1984; Rupert 1988; kuhl et al., 1996). The hydrodynamic volume of PEG is higher than that of a globular protein of the same molecular weight, owing to the strong coordination of water molecules, about 6–7 per monomer unit (Harris, 1991). Interestingly the PEG/protein hydrodynamic volume ratio increases in correlation with the polymer molecular weight (e.g. 10 and 40 kDa PEGs have hydrodynamic volumes corresponding to that of proteins of 65.4 and 670.7 kDa, respectively (Fee and Alstine 2004)). PEG and its conjugates are mainly excreted by kidney clearance and the excretion rate is significantly reduced for molecular weights.
over 40 kDa (Yamaoka et al., 1994; Yamaoka et al., 1995). The toxicology profile of PEG is very safe with evidence of side effects only at very high parental doses, as demonstrated in studies with rabbit where only i.v. injections of very concentrated PEG solutions (PEG 750 Da; LD50 10 g/kg) caused the formation of vacuoles in the epithelium cells of proximal renal tubules (Fruijtier-Polloth, 2005). The amounts of PEG injected with a PEG–protein conjugate are well below the concentrations used in the aforementioned study, thus avoiding concerns, although vacuolation of renal cortical tubular epithelium was observed in rats after a three months treatment with PEGylated tumor necrosis factor binding protein (Bendele et al., 1998). A thorough investigation demonstrated that in this specific case the side effect was observed only with the administration of the conjugate and not with the free protein or PEG, probably as consequence of a cooperative action. It is worth noting that the lesions were partially reversible after two months and did not correlated with any renal dysfunction or changes in urinalysis parameters. Transient vacuoles were also observed in the liver, kidney renal tubules and bone marrow macrophages of monkeys administered with PEG–hemoglobin (Young et al., 2007). Likewise to the aforementioned case, hemoglobin had an active role in determining the localization of vacuoles owing to its clearance pathway: the sequestration by the reticulo-endothelial system (Shum et al., 1996; Conover et al., 1996). In the design of any new PEGylation project, several parameters have to be considered. The appropriate molecular weight of PEG can be easily estimated based on the size of the given protein and the desired in vivo half-life increase, thanks to the support of the many conjugation examples reported in literature, some of them review in Ref. (Maggon, 2007). The election of the activated PEGylating agent and the coupling method are less straightforward and of paramount relevance to ensure a high retention of protein activity by pursuing the polymer coupling to those amino acids not essential for the biological function while, at the same time, shielding the amino acid sequences that are epitopes for antibodies or sensitive to proteolytic degradation. Consequently, a case-by-case approach is necessary to achieve the desired outcomes. The epsilon amino group of lysine is the most exploited in PEGylation by using activated PEG carboxylates or carbonates. This simple chemistry unfortunately faces the common problem of low selectivity in that lysines are a well-represented amino acid in proteins. The rate of amino
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coupling might greatly vary on the basis of both reactivity of the PEGylating agent and nucleophilicity and exposure of the protein amino groups. Usually, quite complex mixtures of isomers are obtained with this random coupling, thus affecting the batch homogeneity, the batch-to-batch reproducibility and the characterization and purification steps. The reactivity of a given PEGylating agent, for example a N-hydroxysuccinimide activated carboxyl PEG, can vary significantly on the basis of the neighboring substituent groups of the reactive moiety (Robert et al., 2002; Pasut et al., 2007). A certain degree of amino-selectivity might be achieved by increasing the steric hindrance of PEG. For example, branched PEGs have a reduced accessibility to buried sites thus restricting the number of potential isomers. The branched form of PEG is also able to shield better a protein with respect to a linear PEG, thus consequently reaching a better immunogenicity reduction and a greater stability towards proteolysis (Monfardini et al., 1995). For further details on the basic chemistries of PEGylating agents and the unique properties of PEG the reader is referred to other dedicated reviews and books (Harris and Chess, 2003; Veronese and Harris, 2002; Harris and Veronese, 2003; Veronese and Harris, 2008; Veronese, 2009).
Introduction

Fig. 1.3 Steps of antibody elicitation potentially affected by PEGylation for the reduction of protein immunogenicity adopted from (Pasut and Veronese; 2009).

1.11 PEGYLATED NANOPARTICLES

Block copolymers with amphiphilic character, having a large solubility difference between hydrophilic and hydrophobic segments, have a tendency to self-assemble into micelles in a selective solvent (Moffitt et al., 1996; Munk et al., 1998; Tuzar and Kratochvil 1993). In an aqueous solution, micelles with core–shell structure are formed through the segregation of insoluble hydrophobic blocks into the core, which is surrounded by a shell composed of hydrophilic blocks. This self-assembling property of amphiphilic block copolymers provides their high utility in the biomedical field as drug carriers, surface modifiers, and colloidal dispersants (Kataoka et al., 2001; Cammas-Marion et al., 1999; Kramarenko et al., 1999; Antonietti and Goltner, 1997)
1.11.1 Polymeric micelles for drug delivery

Drug targeting for efficient accumulation in body is often hampered by the rapid recognition of carrier system by the reticuloendothelial system (RES) and by the subsequent kidney and/or hepatic elimination. Moreover, for modulated drug delivery to solid tumors, which locate outside the blood compartment, the carrier is required to exhibit not just a sufficient half-life in the blood compartment, but also the capability of extravasation at the tumor site. Recent developments led to the design of drug carriers with prolonged circulation in the vascular (Kataoka et al., 1993). Cancer chemotherapy may cause severe effects, leaving patients under extreme distress.

To overcome this problem, an interest has been raised in the application of block copolymer micelles as novel carrier systems for anticancer agents because of the high drug-loading capacity of the inner core as well as of the unique disposition characteristics in the body (Kataoka et al., 1993; Kataoka, 1994; Yokoyama et al., 1991; Yokoyama et al., 1990). Compared to surfactant micelles, polymeric micelles are generally more stable, with a remarkably lowered critical body are often hampered by the rapid recognition of micelle concentration (CMC), and have a slower rate of dissociation, allowing retention of loaded drugs for a longer period of time and, eventually, achieving higher accumulation of a drug at the target site (Kataoka et al., 1993). Polymeric micelles have a size of 30–50 nm in diameter, ranging closely to that of viruses, and apparently, this size range is favorable for extravasation to achieve so-called enhanced permeation retention effect (EPR effect) (Matsumura and Maeda, 1986).

![PEGylated Nanoparticles showing core and surrounding hydrophilic atmosphere.](image-url)
1.12 WHAT ARE TARGETED PEGYLATED NANOPARTICLES (TPNPs) ?

Despite the benefits that nanoparticles have rendered to medicine, some applications remain challenging; for instance, in vivo real-time monitoring of cellular events, specific targeting to the action site or efficient drug delivery inside the target cell. In this context, the design of targeted, shielded (PEGylated) nanoparticles (TPNPs) could significantly improve already existing nanoparticle characteristics and help to surmount these challenges. Whereas monofunctional nanoparticles provide a single function, they can only transport drugs but does not have the inherent property to distinguish between healthy and unhealthy cells or tissues. Targeted, PEGylated nanoparticles combine different functionalities in a single stable construct. For example, a core particle could be linked to a specific targeting function that recognizes the unique surface signatures of their target cells. Simultaneously, the same particle can be modified with an imaging agent to monitor the drug transport process, a function to evaluate the therapeutic efficacy of a drug, a specific cellular penetration moiety and a therapeutic agent (Torchilin, 2006) (Figure 1.5).

The diagnostic and/or therapeutic objectives of a targeted, PEGylated nanocarrier system dictate the design of the formulation. It is observed that the literature obviates there are many different types of nanocarrier formulations for the diagnosis, imaging, and treatment of a wide spectrum of diseases. These targeted, PEGylated nano carriers share three main design components: platform (core) material, encapsulated payload/biologically active agents, and targeting/surface properties (Fig. 1.5).

**Fig. 1.5** Targeted, shielded carriers with their design components.
Introduction

PEG Anchoring chains
Active Targeting Ligand
Nanocarriers (nanoparticles, liposomes etc) Encapsulated Drug

Fig. 1.6 Nanocarrier (encapsulated drug) with PEG attached with ligand.

1.13 ADVANTAGES OF TPNPs

1. Through PEGylation we can maintain drug levels in the blood, therefore can improve specificity.

2. Through attaching specific targeting moiety (like folic acid, galactose, heparin), we can increase site specificity and thus reduce toxicity.

3. Through specific ligands the intracellular penetration can be achieved.

4. Through stimulus-sensitive polymers or through the potosytosis, the cytosolic drug release can be achieved and thus we can -
   a. Overcome the Multi-drug resistance.
   b. Reduce toxicity.

5. In targeted, PEGylated Nanoparticles the contrasting agents can be incorporated to localize the in vivo accumulation of nanoparticles.
Table 1.3 OTHER METHODS OF NANOPARTICLES PREPARATION
(Mohanraj *et al.*, 2006)

<table>
<thead>
<tr>
<th>Technique</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emulsion polymerization</td>
<td>Poly(alkyl methacrylate)</td>
</tr>
<tr>
<td></td>
<td>Poly(alkyl cyanoacrylate)</td>
</tr>
<tr>
<td></td>
<td>Poly(styrene)</td>
</tr>
<tr>
<td></td>
<td>Poly(vinylpyridine)</td>
</tr>
<tr>
<td></td>
<td>Poly(acroleine)</td>
</tr>
<tr>
<td></td>
<td>Poly(glutaraldehyde)</td>
</tr>
<tr>
<td>Interfacial polymerization</td>
<td>Poly(alkyl Cyanoacrylate)</td>
</tr>
<tr>
<td></td>
<td>Poly(lysine) derivative</td>
</tr>
<tr>
<td>Emulsification evaporation</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td></td>
<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td></td>
<td>Poly(1-caprolactone)</td>
</tr>
<tr>
<td></td>
<td>Poly(b-hydroxybutyrate)</td>
</tr>
<tr>
<td></td>
<td>Ethyl cellulose</td>
</tr>
<tr>
<td>Solvent displacement</td>
<td>Poly(alkyl methacrylate)</td>
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<tr>
<td></td>
<td>Poly(lactide acid)</td>
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<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td></td>
<td>Poly(1-caprolactone)</td>
</tr>
<tr>
<td>Salting out</td>
<td>Cellulose acetate phthalate</td>
</tr>
<tr>
<td></td>
<td>Poly(alkyl methacrylate)</td>
</tr>
<tr>
<td></td>
<td>Ethyl cellulose</td>
</tr>
<tr>
<td></td>
<td>Poly(lactide acid)</td>
</tr>
<tr>
<td></td>
<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>Emulsification diffusion</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td></td>
<td>Poly(1-caprolactone)</td>
</tr>
<tr>
<td>Desolvation, denaturation</td>
<td>Albumin</td>
</tr>
<tr>
<td></td>
<td>Casein</td>
</tr>
<tr>
<td></td>
<td>Gelatin</td>
</tr>
<tr>
<td></td>
<td>Alginate</td>
</tr>
</tbody>
</table>
1.14 TARGETED PEGYLATED NANOPARTICLES (TPNPs) INCLUDES

Targeted PEGylated Nanoparticle includes following aspects-

1.14.1 PEGylation –

Despite advantageous effect of nanoparticles, a drug delivery device must be present in the circulation for long enough time to reach its intended target tissue. Plasma proteins, known as opsonins, can bind circulating drug delivery devices, including nanocarriers, and remove them from the circulation within seconds to minutes through the reticulo-endothelial system (RES) (Owens et al., 2006). Imparting a stealth-shielding on the surface of these drug delivery systems prevents opsonins from recognizing these particles, thereby limiting phagocytosis by the RES cells and increasing the systemic circulation time from minutes to hours or even days (Owens et al., 2006). Poly (ethylene glycol) (PEG) (known as poly (ethylene oxide) (PEO) when the molecular weight is greater than 20 kDa) modification has emerged as a common strategy to ensure such stealth shielding and long-circulation of therapeutics or delivery devices. PEG-modification is often referred to as PEGylation, a term that implies the covalent binding or non-covalent entrapment or adsorption of PEG onto an object.

PEG has a general structure of HO–(CH₂CH₂O)ₙ–CH₂CH₂–OH, encompassing a polyether backbone that is chemically inert, with terminal hydroxyl groups that can be activated for conjugation to different types of polymers and drugs.

The protective (stealth) action of PEG is mainly due to the formation of a dense, hydrophilic cloud of long flexible chains on the surface of the colloidal particle that reduces the hydrophobic interactions with the RES. The tethered and/or chemically anchored PEG chains can undergo spatial conformations, thus preventing the opsonization of particles by the macrophages of the RES, which leads to preferential accumulation in the liver and spleen. PEG surface modification, therefore, enhances the circulation time of molecules and colloidal particles in the blood (Veronese et al., 2005; Kommareddy et al., 2005; Gref, 1994). In addition, PEG surface modification may also increase the hydrodynamic size of the particle decreasing its clearance, a process that is dependent on the molecular size as well as particle volume. PEG-modification of PLGA nanocarriers improved circulation time, whereby only 5% of unmodified particles
remained in the circulation within 5 min of administration, but as much as 25% of PEG (molecular weight of 5,000 Da) remained circulating.

**Advantages of PEGylation -**

1. Enhanced circulation time of carriers in systemic circulation.
2. Decrease in body clearance.
3. Passive targeting can be possible.
4. Active targeting can be possible.
5. Intracellular delivery of carriers can be possible.
6. Further Surface modification, and attachment of ligands can be possible.

### 1.14.2. LIGAND

#### 1.14.2.1 FOLIC ACID AS TARGETING MOIETY

![Folic Acid Structure](image)

**Fig. 1.7 Structure of Folic acid**

**Molecular formula-** $\text{C}_{19}\text{H}_{19}\text{N}_{7}\text{O}_6$

Folic acid (also known as Vitamin M and Folacin) and Folate (the anionic form) are forms of the water-soluble Vitamin $\text{B}_9$. These occur naturally in food and can also be taken as supplements. Folate gets its name from the Latin word folium ("leaf").

The folic acid (FA) enters cells through a carrier protein, termed the reduced folate carrier, or via receptor-mediated endocytosis facilitated by the folate receptor (FR). Because folate–drug conjugates cannot serve as substrates of the former, they penetrate cells exclusively via FR-mediated endocytosis (reviewed by Reddy and Low (Reddy et
The receptor for folate, also known as folate-binding protein, is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein with an average molecular weight of 38 kDa. It mediates the cellular uptake of folate and related coenzymes via receptor-mediated endocytosis occurring at non-coated membrane regions termed caveolae (Rothberg et al., 1990). Ligand binding to folate receptors stimulates their endocytic uptake by a clathrin-in-dependent pathway (potocytosis) that has been exploited to negotiate the non-specific uptake of macro molecules and particulate drug delivery systems (Anderson, 1993). In the potocytosis model of folate internalization, folate is proposed to bind receptors clustered around caveolae, i.e. specialized regions of the cell membrane enriched in cholesterol and other GPI anchored proteins. These caveolae then invaginate, forming a membrane-linked or cabled compartment within the cytoplasm. An integral proton gradient is then proposed to facilitate the release of the bound folate, allowing its movements across the caveolae membrane by an integral membrane anion carrier (Lisanti et al., 1994).

1.14.2.2 HEPARIN AS TARGETING MOIETY

Major repeating disaccharide of heparin

Minor repeating disaccharide of heparin

Fig. 1.8 Major and minor repeating disaccharide sequences of heparin

Molecular Formula- \( \text{C}_{12}\text{H}_{19}\text{NO}_{20}\text{S}_{3} \)

Heparin is a highly sulfated, anionic polysaccharide composed of repeating glucosamine and uronic acid residues. This was discovered in 1916, and has been used clinically as an anticoagulant since 1935; it is second only to insulin as a natural
therapeutic agent (Sundaram et al., 2003). Heparin is best known for its anticoagulant properties but also interacts with a variety of proteins that have heparin-binding domains including various growth factors which enables the growth factors to crosslink their receptors and to be protected from proteolytic degradation. The heparin is linear polysaccharides consisting of uronic acid- (1 4)-D-glucosamine repeating disaccharide subunits. The uronic acid of the uronic acid- (1 4) - D-glucosamine repeating disaccharides may be either α- D-iduronic acid (IdoA) or β- D -glucuronic acid (GlcA). Variable patterns of substitution of the disaccharide subunits with N-sulfate, O-sulfate and N-acetyl groups give rise to a large number of complex sequences. The ligand is structurally the most complex members of the glycosaminoglycan (GAG) family of polysaccharides. This is expressed in connective-tissue type mast cells (Casu, 1989).

Heparin plays a key role in angiogenesis, viral invasion, and tumor metastasis (Rabenstein, 2002). Heparin is biosynthesized as heparin proteoglycan, which consists of a unique core protein (serglycin) to which are covalently attached multiple heparin polysaccharide chains.

1.14.2.3 GALACTOSE AS TARGETING MOIETY

![Galactose](image)

Galactose

**Fig. 1.9 Structure of Galactose**

**Molecular formula**- $C_6H_{12}O_6$

Galactose (Gal) is a type of sugar that is less sweet than glucose. It is considered a nutritive sweetener because it has food energy. Its name comes from the Ancient Greek word for milk, γάλακτος (galaktos). It is an epimer of glucose.

Galactan is a polymer of the sugar galactose. It is found in hemicellulose and can be converted to galactose by hydrolysis. Galactose solubility in water is 68.30 grams per
100 grams of water at 20–25°C. Among the cellular targets for ligands, the asialoglycoprotein-receptor (ASGP-R) is expressed exclusively on hepatocytes (Stockert, 1995): ASGPR naturally binds and internalises the terminal galactose-binding asialoglycoprotein. Thus, galactose molecules grafted at the surface of a carrier could provide an active targeting. This can be useful for efficient targeting of the liver, and a consequent secretion of therapeutic gene products into systemic circulation.

The galactose ligand is used to target the asialoglycoprotein receptor (ASGPR) that is expressed on hepatocytes in hopes that it was still highly expressed on primary liver cancer cells. However, because the ASGPR is expressed on healthy hepatocytes, the targeted nanoparticles accumulated in normal liver as well as in the tumor (James et al., 2008). The receptor–ligand interaction was known to be showing a significant ‘cluster effect’ in which a multivalent interaction results in extremely strong binding of ligands to the receptors (Lee et al., 1983).

**Advantages of Ligand attachment-**

1. As targeting moiety.
2. To avoid non-specific attacks on normal tissues.
3. Ligand-Drug conjugate can be easily designed.
4. High affinity of Ligand to receptor facilitates internalization via potosytosis/receptor mediated endocytosis.
5. Deeper penetration can be achieved.

**1.14.3 Capability to overcome Multi – Drug Resistance-**

An alternative strategy for overcoming drug resistance is based on new drug delivery systems to achieve selective drug accumulation in tumor tissues, tumor cells, or even compartments of tumor cells. Liposomal carriers have become clinically accepted in cancer treatment, and as such are examples of delivery systems that can enhance the utility of anticancer drugs. For example, long circulating liposomes and other macromolecular carriers can increase drug deposition in solid tumors, which may help to overcome drug resistance. Other liposome strategies include modifications for controlled release, which may increase drug bioavailability; and ligand-targeted liposomes, such as
immunoliposomes, which can internalize in tumor cells for intracellular drug delivery and maximal drug efficacy (Mamota et al., 2003).

Combination of liposome pH-sensitivity and specific ligand targeting for cytosolic drug delivery utilizing decreased endosomal pH values was described for both folate and Tf-targeted liposomes (Xu et al., 2002).

**Multi-Drug resistance can be overcome by-**

1. By achieving selective drug accumulation in tumor cell.
2. By the use of long circulating carriers (PEGylated).
3. By modifying surface property to achieve cytosolic drug delivery.
4. By using specific ligands having deeper penetration on cell.
5. PEG itself work as a Class III glycoprotein pump inhibitor, so it will inhibit the pumps involved in efflux mechanism.
1.15 DRUG PROFILE: CISPLATIN

Drug selected for present study is “Cisplatin” which is widely used in the management of cancer chemotherapy.

Table 1.4 PHYSICOCHEMICAL DESCRIPTION OF DRUG.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
<td>(NH₃)₂PtCl₂</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>300.1g/mol</td>
</tr>
<tr>
<td>Structure</td>
<td>(cis- diaminodichloroplatinum (II))</td>
</tr>
<tr>
<td>Definition</td>
<td>According to B.P. (2004), it contains not less than 99.0% and not more than the equivalent of 102.0%w/w of cisplatin. It belongs to the category of antineoplastic drug.</td>
</tr>
<tr>
<td>Physical Characteristics</td>
<td>Yellow crystalline powder, odorless, Decomposes with blackening at about 270°C.</td>
</tr>
<tr>
<td>Solubility Parameters</td>
<td>:</td>
</tr>
</tbody>
</table>
**Introduction**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Solvents</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>DMSO (1g in 80 ml)</td>
<td>Soluble</td>
</tr>
<tr>
<td>2.</td>
<td>DMF (1g in 55ml)</td>
<td>Sparingly Soluble</td>
</tr>
<tr>
<td>3.</td>
<td>*Water (1g in 170ml)</td>
<td>Slightly Soluble</td>
</tr>
<tr>
<td>4.</td>
<td>Ethanol, Ether, Chloroform</td>
<td>Practically Insoluble</td>
</tr>
</tbody>
</table>

(Florey, 1973) * Solubility in water depends on time.

<table>
<thead>
<tr>
<th>Melting Range</th>
<th>:</th>
<th>270 to 272°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage</td>
<td>:</td>
<td>Store in a tightly closed, light resistant container</td>
</tr>
<tr>
<td>Caution</td>
<td>:</td>
<td>cisplatin is irritant, so avoid contact with skin and mucous membranes (Martindale, 2005)</td>
</tr>
</tbody>
</table>

**1.16 PHARMACOLOGY**

**1.16.1 Mechanism of Action** (Goodman & Gilman, 1992)

Cisplatin appears to enter cells by diffusion. The chloride atoms may be displaced directly by reaction with nucleophils, hydrolysis of chlorides is probably responsible for formation of the activated species of the drug. The platinum complexes can react with DNA forming both intrastrand and interstrand cross-links. Most notable among the DNA changes are the 1, 2-intrastrand cross-links with purine bases. The N7 of guanine is a particularly reactive site, leading to platinum cross-links between adjacent guanines on the same DNA strand; guanine-adenine cross-links also readily form and may be critical.
to cytotoxicity. The formation of interstrand cross-links is less favored. The covalent binding of protein to DNA has also been demonstrated. Cisplatin crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible. Cisplatin with regard to phase of cell cycle shows effect on cross-linking during the S-phase.

1.16.2 Absorption, Fate and Excretion (Goodman & Gilman, 1992)

After intravenous administration, cisplatin has an initial plasma elimination half-life of 25 to 50 minutes; concentrations of total (bound and unbound) drug fall thereafter, with a half-life of 24 hours or longer. More than 90% of the platinum in the blood is covalently bound to plasma proteins. The unbound fraction, composed predominantly of parent drug, is cleared within minutes. High concentrations of cisplatin are found in the tissues of the kidney, liver, intestine, and testes, but there is poor penetration into the CNS. Only a small portion of the drug is excreted by the kidney during the first 6 hours; by 24 hours up to 25% is excreted and by 5 days up to 43% of the administered dose is recovered in the urine, mostly covalently bound to protein and peptides. Biliary or intestinal excretion of cisplatin is minimal.

1.16.3 Pharmacokinetic Parameters (Goodman & Gilman, 1992)

Following intravenous administration, cisplatin concentrates in the liver, kidneys and the large and small intestines. Penetration into CNS (central nervous system) is poor. It is distributed into breast milk. The chlorine atoms in cisplatin undergo chemical displacement reactions with water and sulfhydryl groups (for example, on proteins) rather than undergoing enzyme–catalysed metabolism. Excretion is mainly in urine but is incomplete and prolonged; up to 50% of a dose has been reported to be excreted in urine over 5 days and platinum may be detected in tissue for several months afterwards. There is some excretion in faeces via bile. Cisplatin is well absorbed following intraperitoneal administration.
1.16.4 Clinical Toxicity (Goodman & Gilman, 1992)

The major toxicity caused by cisplatin is dose related cumulative impairment of renal tubular function (Nephrotoxicity), this usually occurs during second week of therapy. Ototoxicity caused by cisplatin is unaffected by diuresis and is manifested by tinnitus and high-frequency hearing loss. The ototoxicity can be unilateral or bilateral, tends to be more frequent and severe with repeated doses and may be more pronounced in children. Marked nausea and vomiting occur in almost all patients. Cisplatin causes mild-to-moderate myelosuppression with transient leukopenia and thrombocytopenia. Anemia may become prominent after multiple cycles of treatment. Electrolyte disturbances including hypomagnesemia, hypocalcemia, hypokalemia and hypophosphatemia are common. Hypocalcemia and hypomagnesemia secondary to renal electrolyte wasting may produce tetany if untreated.

1.16.5 Therapeutic Uses (Goodman & Gilman, 1992)

Cisplatin, in combination with bleomycin, etoposide, ifosfamide or vinblastine cures 90% of patients with testicular cancer. Used with paclitaxel, cisplatin induces complete response in the majority of patients with carcinoma of the ovary. Cisplatin produces responses in cancers of the bladder, head, neck, cervix, endometrium, all forms of carcinoma of the lungs, anal, rectal and neoplasms of childhood. Interestingly, the drug also sensitizes cells to radiation therapy and enhances control of locally advanced lungs, esophageal, head and neck tumors when given with irradiation.
1.16.6 Drug Interaction (Martindale, 2005)

Use of cisplatin with other nephrotoxic or ototoxic drugs may exacerbate the adverse effects of cisplatin. The ototoxicity of the cisplatin was reportedly enhanced by ifosfamide. The nephrotoxicity developed when cisplatin given along with antihypertensive therapy.

1.16.7 Dose and Route of Administration (Martindale, 2005)

Cisplatin is given by I.V. infusion in sodium chloride 0.9% or sodium chloride and glucose. Cisplatin Up to 120 mg/m² body–surface area by intravenous infusion as a single dose, or 15 to 20 mg/m² by infusion daily for 5 consecutive days, every 3 to 4 weeks. Lower dose are generally used for combination chemotherapy regimens than for single agent therapy.

1.16.8 Marketed Preparations

Table 1.6 showing marketed preparation of Cisplatin

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Brand Name</th>
<th>Dosage Form</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BLASTOLEM</td>
<td>Injection</td>
<td>ELDER</td>
</tr>
<tr>
<td>2.</td>
<td>CISPLAN</td>
<td>Injection</td>
<td>NATCO</td>
</tr>
<tr>
<td>3.</td>
<td>CISPLAT</td>
<td>Injection</td>
<td>BIOCHEM</td>
</tr>
<tr>
<td>4.</td>
<td>CISPLATIN</td>
<td>Injection</td>
<td>PHARMACIA</td>
</tr>
<tr>
<td>5.</td>
<td>PRATICIS</td>
<td>Injection</td>
<td>GERMAN</td>
</tr>
</tbody>
</table>

1.16.9 Identification of Drug

A. To 2ml of dil. NaOH solution added 50mg cisplatin in a glass dish. evaporate to dryness. Dissolved it in mixture of 0.5ml of HNO₃ and 1.5ml of HCl. Evaporate to dryness and then dissolved in 0.5ml of H₂O and added 0.5ml of NH₄Cl solution, resulted a yellow crystalline precipitate formed (Florey 1973).
B. The UV spectrum of cisplatin in HCl (0.1N) in the range of 220 nm to 400 nm exhibit, absorption maximum at 301 nm I.P., (1996).

1.17 ESTIMATION OF DRUG

1.17.1 Analytical Techniques

An analytical technique is a method that is used to determine the concentration of a chemical compound or chemical element. There are a wide variety of techniques used for analysis, from simple weighing (gravimetric) to titrations (titrimetric) to very advanced techniques using highly specialized instrumentation. The most common techniques used in analytical chemistry are the following:

- Titrimetry, based on the quantity of reagent needed to react with the analyte
- Electroanalytical techniques, including potentiometry and voltammetry
- Spectroscopy, based on the interaction of the analyte with electromagnetic radiation
- Chromatography, in which the analyte is separated from the rest of the sample so that it may be measured without interference from other compounds

There are many more techniques that have specialized applications, and within each major analytical technique there are many applications and variations of the general techniques.

1.17.1.1 Spectrophotometric Methods

The IP (1996) recommends an ultraviolet spectrophotometric method for the determination of cisplatin in 0.1N HCl solution. It exhibits adsorption maxima at 301 nm. Anilanmert et al., (2001) developed a method based on the measurement of the absorbance of the reaction product of the cisplatin and o-phenylenediamine, which showed a maximum absorbance at 705 nm. Raghavan and Mulligan, (2000) described
atomic absorption method in which cisplatin was reacted with diethyldithiocarbamic acid (DDTC), sodium salt, to yield a platinum-DDTC (Pt-DDTC) complex. The Pt-DDTC chelate was extracted into methylene chloride, the extract was mixed with acetonitrile and the platinum content was then determined using a Zeeman atomic absorption spectrophotometer.