Chapter 2  Literature Review

2.1 The cancer

Cancer or malignant neoplasm is a disease entity characterized by uncoordinated and accelerated cell growth and proliferation in the human body. Such abnormal cellular growth often results in formation of an abnormal tissue mass (tumour) that brings detrimental effects to the host’s body. Cells of cancer tissue may possess features that resemble their normal counterparts, although in certain severe cases, cancer cells may lose all their differentiating features to the extent that their tissue of origin cannot be identified (Bass, 1997). Different from the benign neoplasm that assume localized and confined growth (normally in a fibrous capsule), and are non-invasive to the surrounding tissue and non-metastatic, cancers take rapid and invasive growth into surrounding tissue and potentially spread to other parts of the host’s body (metastasis). The end results are often fatal.

Throughout the world, cancer has been well recognised as the second major life threatening disease after cardiovascular diseases. Annually, about 10.9 million people worldwide are diagnosed with cancer, whereby 45% of the cases are found in Asia. Cancer death is about 6.7 million people per year, which is 12% of total death worldwide (Parkin et al., 2005). Among these, cancers of the lung, breast, bowel, stomach and prostate are the commonest cancers found and these cancers account for 12, 11, 9, 9 and 6% of annual total cancer cases diagnosed respectively (Parkin et al., 2005). Lung cancer is the commonest cause of death from cancer (accounts for 18% of annual cancer death), followed by stomach, liver, bowel and breast cancers (10, 9, 8 and
6% of annual cancer deaths respectively) (Parkin et al., 2005). In Malaysia, cancer is the second leading cause of death with estimated 40,000 new cancer cases registered annually (MAKNA, 2006). Recent statistics showed that lung and breast cancers are the commonest cancers found in the male and female Malaysians (13.8 and 31.0% of all cancers in male and female Malaysians respectively) (Lim & Yahaya, 2004).

### 2.2 Types of cancer

In clinical practice, cancers are typed according to a few criteria to ease the disease monitoring, prognosis estimation and treatment. Cancers are divided according to cellular origin into 3 main types, i.e. carcinomas (cancers of the epithelial cells), sarcomas (solid tumours of the connective tissue) and leukaemias (cancers of the blood and lymphatic systems) (ACS, 2005; Bishop, 1999). Of the three, carcinomas constitute approximately 90% of all human cancer cases, leukaemias and lymphomas 8% while sarcomas cases are rare. Cancers can also be classified according to the following ways (ACS, 2005; Bishop, 1999):

- the site of origin, for example, breast or lung carcinomas are cancers originating from the breast or lung,
- the cell type, for example, adenocarcinoma is carcinoma of glandular epithelium, rhabdomyosarcoma is sarcoma of muscle cells and acute lymphocytic leukaemia is leukaemia of immature lymphocytes,
- the cell morphology, for example, large and small cell carcinomas of lung,
- the resemblance of the cancer cells to embryonic tissues, for example, neuroblastoma represents childhood neuronal cell cancer and retinoblastoma is a childhood eye cancer.
2.3 Causes of cancer

The development of cancer in normal body tissue is referred to as carcinogenesis. In general, cancer arises in tissue as a result of genetic mutation in the once normal cell. Genetic mutation that can lead to carcinogenesis can be induced by various events and mutagens, or simply inherited from one’s ancestors, as described in the following subchapters.

2.3.1 Chemically induced cancerous genetic mutation

Genetic mutations that can lead to carcinogenesis can be induced by chemicals. These chemicals are often referred as carcinogens. Most carcinogens can be classified under 6 main categories, as listed in Table 2.3.1 (Gooderham, 2002; Poirier, 2004).

Table 2.3.1 Carcinogens categories and examples

<table>
<thead>
<tr>
<th>Carcinogen categories</th>
<th>Examples</th>
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<tbody>
<tr>
<td>polycyclic hydrocarbons</td>
<td>benzo(a)pyrene and dibenz (a,h) anthracene</td>
</tr>
<tr>
<td>aromatic amines</td>
<td>benzidine, 2-naphtylamine and 4-aminobiphenyl</td>
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<tr>
<td>aminoazo compounds</td>
<td>dimethylaminoazobenzene and o-aminозотолуене</td>
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<tr>
<td>N-nitroso compounds</td>
<td>nitrosamine and nitrosourea</td>
</tr>
<tr>
<td>alkylation agents</td>
<td>vinyl chloride, ethylene oxide and sulphur mustard</td>
</tr>
<tr>
<td>carcinogenic natural products</td>
<td>aflatoxin, safrole and pytolizidine alkaloids</td>
</tr>
<tr>
<td>inorganic carcinogens</td>
<td>cadmium, chromium, nickel and asbestos</td>
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Chemical carcinogens (or their electrophilic derivatives) induce genetic mutation by first conjugating themselves to the electron rich atoms in the DNA, for
example, the amino nitrogen of the nucleotide bases (Gooderham, 2002; Poirier, 2004). Such conjugations eventually result in various forms of DNA damage, including the formation of DNA adduct, DNA methylation, single and double strand breaks, nucleotide crosslinking, hydroxylated / missing bases, etc. (Gooderham, 2002; Poirier, 2004). These DNA damages can be made permanent into carcinogenic mutations (e.g. through wrong base pairing and error DNA template reading by DNA polymerase during DNA replication) if they are not repaired in time / fail to be repaired by the cellular DNA repair mechanisms.

2.3.2 Radiation induced cancerous genetic mutation

High energy radiations, especially ultraviolet (UV) radiation and ionizing radiations are well known to be able to induce DNA mutations. UV radiations, typically UVB radiation (radiation wavelength ranging from 280 to 315 nm, from sunlight) were capable of triggering pyrimidine base dimerization along a DNA strand, which may lead to DNA replication error and irreversible DNA mutation (Brash et al., 1991; Leffell and Bash, 1996). Generally, the UV radiation induced carcinogenic DNA mutations (typically p53 gene loss of function mutation) commonly occur to the cells of the body that have direct contact or are nearer to the outer environment, e.g. cells of the epidermis and dermis (Boice et al., 1997; Little, 2000; Ziegler et al., 1994).

On the other hand, high energy ionizing radiations, for example γ ray, x-ray, α- and β-radiations generate hydroxyl free radicals within the normal tissue (Little, 2000). The formed free radicals are capable of altering / removing the DNA bases on / from the DNA or cause single and double DNA strand breaks (Little, 2000; Ullrich & Ponnaiya, 1998), leading to carcinogenic gene mutation events such as DNA replication errors
(e.g. wrong base pairing and error DNA template reading by DNA polymerase), large scale DNA sequence deletions, DNA sequence rearrangements and chromosomal translocations (Little, 2000; Ullrich & Ponnaiya, 1998). Overdose exposure to these ionizing radiations is usually occupational or following a catastrophic event (e.g. nuclear explosion) (Boice et al., 1997; Little, 2000). However, accumulation of α-radiation emitting substance e.g. polonium may occur in the lung through chronic inhalation of tobacco smoke (Winters & Di Franza, 1982).

2.3.3 Infectious agent induced cancerous genetic mutation

Infectious agents such as parasites, bacteria and viruses have been found to contribute to carcinogenic genetic mutation induction in three ways. Firstly, by debilitating or disabling the body’s immune system, infectious agent such as HIV may open ways for other opportunistic infectious agents e.g. KSHV, EBV, HPV and hepatitis virus B and C to induce cancerous genetic mutation (Boshoff & Weiss, 2002). Secondly, infectious agents for example Hepatitis B and C viruses and Helicobacter pylori may induce chronic inflammation in the infected tissue (e.g. liver and stomach), causing prolonged and elevated cell proliferation (as part of the cellular repair mechanism) and elevated production and free radicals release by lymphocytes and macrophages (as an anti-infection measure) at the inflamed region. Intense cell replication activity in the presence of DNA damaging free radicals may significantly increase the occurrence of genetic mutation and carcinogenesis, for example liver and stomach cancer (Coussens & Werb, 2002; Peek & Blaser, 2002). Thirdly, infectious agents may initiate carcinogenesis by directly stimulating the proliferation of the infected cells (Griffin, 2002; Parsonnet, 1999). These infectious agents, mainly viruses stimulate cell proliferation by producing growth signalling proteins (for example, v-src tyrosine...
kinase by Rous sarcoma virus), disabling the gatekeepers or cell replication controllers (for example, the E6 and E7 proteins by the human papilloma virus, which bind and disable the p53 and Rb proteins respectively) or by altering the expression of the gene that codes for the cell signalling pathway components (for example overproduction of normal Myc protein due to insertional mutagenesis induced by the insertion of the avian leucosis viral DNA into region near to the MYC gene of the cell) (Griffin, 2002; Parsonnet, 1999; zur Hausen, 1996). Accelerated and uncontrolled cell proliferation may often lead to DNA replication errors and gene mutations, including the carcinogenic ones.

2.3.4 Heredity acquired cancerous genetic mutation

DNA mutations inherited from the family lineage may predispose a person to carcinogenesis. In the earlier members of a family, chemical, radiation or infectious agent may induce DNA damages. These DNA damages can be made permanent into DNA mutations and be inherited by the offspring as a result of inefficiency in DNA repair or escape of cells from apoptotic death. The genetic mutations found in heredity induced carcinogenesis can be roughly divided into three main groups. In one is loss of function mutation of the gatekeeper genes or caretaker genes in the second group, and the third group having gain of function mutation of the proto-oncogene. The loss of function mutation of the gatekeeper gene (for example APC, RB, p53, WT, and others) or the caretaker genes (for example ATM, BRCA 1 & 2, XPA-G, and others) may lead to impairment of cell apoptosis and proliferation control and defective DNA damage repair respectively. A number of cancers or cancer related diseases such as familial adenomatous polyposis, familial retinoblastoma, Li-Fraumeni syndrome and Wilms tumour (for respective gatekeeper gene examples), as well as Ataxia telangiectasia,
familial breast cancer and Xeroderma pigmentosa (for respective caretaker gene examples) may have developed in the carriers of these defective genes (Cleaver, 2005; Evans, 2002; King et al., 2003; Kinzler & Vogelstein, 1997; Shiloh, 2003). Meanwhile, the gain of function mutation may turn a proto-oncogene (for example, RET gene) into an oncogene that produces abnormal proteins that initiate uncontrolled cell proliferation and carcinogenesis (e.g. mutant RET protein in the case of the multiple endocrine neoplasia type 2) (Marx, 2005). Depending on the type of mutations inherited, a person may face different levels of risk (dominant or recessive) in developing cancer. In the cases of the dominant cancer risk syndromes such as familial retinoblastoma, familial adenomatous polyposis and Li-Fraumeni syndrome, inheritance of a single lost of function mutation in the tumour suppressor genes (RB, APC and p53 respectively) is sufficient to impose high carcinogenic risk to a person (Evans, 2002; Frank, 2004; Knudson, 1971; Li & Fraumeni, 1982). However, since a normal person always carries two copies of genes for any single genetic trait; a second copy of the gene must become inactivated for the cancer to actually arise in the person, according to Knudson’s 2 hits theory (Knudson, 1971). However in the case of recessive cancer risk syndrome e.g. xeroderma pigmentosum, two defective copies of tumour suppressor gene (for example the XP genes series) needs to be inherited to predispose a person with high carcinogenesis risk (Cleaver, 2005).

2.4 Types of carcinogenic DNA damages and genetic mutations found in cells

In a normal cell, various types of DNA damage, for example depurination, deamination, base replacement / modification / intercalation, pyrimidine dimerization may lead to carcinogenic gene mutations such as point mutation, base mismatch, gene
amplification, chromosomal translocation, local DNA rearrangement, insertional mutation, DNA single or double strand break and gene truncation or deletion (Friedberg, 2003; Rowley, 2001; Schwarb, 1998; Weinberg, 1983). Although there are instances whereby single genetic mutation in cells triggers the development of cancer in normal tissue, the occurrence of cancer is often a result of the complex interactions among a few mutated genes, which at the end of these interactions confer the cancer cells superior growth advantages over the normal cells.

2.5 DNA damage repair and mutation

Cells have gene repair mechanisms that remove deleterious mutations. Mutations involving abnormal bases are corrected by translesion synthesis or excision repair. Translesion synthesis is a mechanism that synthesizes error free new DNA strands (by DNA polymerase η) using the DNA template strand that contains the damage. For example, DNA polymerase η may introduce a correct -A-A- sequence in the new DNA strand in response to the pyrimidine dimer -T=T- on the DNA template strand. Excision repair is a process that removes damaged DNA regions from the DNA strand using repair endonucleases, followed by DNA strand repair and annealing using DNA polymerase and DNA ligase (Friedberg, 2003; Lindahl & Wood 1999). Meanwhile, pyrimidine dimers and other bulkier damage on the DNA strands are removed by nucleotide excision repair (NER), a process that involves recognition of major distortion along the DNA strand by specific proteins, followed by the removal of the distorted DNA region by the NER endonuclease and DNA strand repair and annealing by DNA polymerase and DNA ligase (Friedberg, 2003; Lindahl & Wood 1999). On the other hand, base mismatch is repaired by excising the mismatched region off the new DNA strand followed by strand repair using the original DNA strand as
template, while DNA double strand break is repaired via non-homologous end joining (joining of the ends of 2 DNA fragments using a set of proteins) or homologous recombination (repair of double strand breaks occur on a chromosome using its sister chromosome as a template) (Friedberg, 2003; Lindahl & Wood 1999). However, some of the DNA damages may be made permanent into DNA mutations and are difficult to remove if the damaged DNA is replicated before the repair takes place or the repair mechanism accidentally makes permanent the DNA damage (e.g. in the case of the non-homologous end joining). The formed mutation(s) are proposed as a main contributor to carcinogenesis.

2.6 Overview of carcinogenesis

Carcinogenesis can be divided into 3 stages in general, that is, initiation stage, promotion stage and tumour progression stage (Figure 2.6). Initiation stage covers the period from the occurrence of DNA damage in a normal cell of a particular tissue or organ till the first or first few genetic mutations successfully lodge themselves in the cell and move the cell into a precancerous state (Cohen & Ellwein, 1993).

Subsequently, the precancerous cell will enter into the promotion stage, whereby the cell will go through a series of uncontrolled cell proliferation to produce multiple clones that carry the initial DNA mutations as well as additional DNA mutations (occurs during the rapid and uncontrolled cell proliferation) that confer them growth advantages over the surrounding normal cells in the tissue or organ (Cohen & Ellwein, 1993).
Figure 2.6  Different stages of carcinogenesis

Figure shows the various events that occur in the different stages of the carcinogenesis.
The uncontrolled proliferation of the cells can be of its own initiative under the influence of the oncogenes (e.g. BCR-ABL fusion gene in the case of chronic myelogenous leukaemia, TRK oncogene in the case of thyroid cancer and HRAS oncogene in the case of bladder cancer), or in the absence of functional tumour suppressor genes (e.g. APC gene in the case of familial adenomatous polyposis, BRCA1 & 2 in the case of breast cancer or p53 genes in many types of cancers) (Fearon, 2000; Futreal et al., 2004). Alternately, uncontrolled proliferation of the precancerous cell can also be encouraged by continuous exposure to chemical carcinogens that activate the component of the cell proliferation signalling pathway (e.g. phorbol esters, teleocidin and aplysiatoxin which act on the protein kinase C) or radiation (e.g. chronic exposure of the skin to UVB radiation may lead to the apoptotic death of the cells with radiation induced DNA damage but normal p53 gene, leaving extra space and growth resources for the proliferation of precancerous skin cells that has p53 loss of function mutation) (Brash et al., 1991; Gooderham, 2002; Leffell & Bash, 1996; Luch, 2005). Along with the multiple rounds of uncontrolled proliferation of the precancerous cells, clonal selection occurs whereby the cancer cell clones with faster and more autonomous proliferation traits will deprive the growth of other precancerous clones and normal cells in the same growth region. Eventually at the end of the promotion stage, the dominating clones form a malignant tumour.

Following the promotion stage, the tumour progression stage begins, whereby the cancerous cell population of the newly formed tumour continue to acquire more aberrant traits (e.g. increase in growth and proliferation rate, increase in invasiveness, anchorage independency, drug and immune resistance, ability to metastasize and relocate in the tissue / organ distant from the primary tumour site, ability to induce angiogenesis, immortality, etc) through ongoing genetic mutation. With the acquired
aberrant traits, the cancer cells form a tumour mass that aggressively proliferates and invade the surrounding normal tissue (Cohen & Ellwein, 1993). Upon breaching of the walls of the tissue’s blood vessels, the tumour mass sheds large quantities of cancer cells into the blood circulation, and thus initiates tumour metastasis. Eventually, the metastasis and growth of the cancer cells in the tissue or organs of the body will disrupt and deprive the normal growth and function of the body tissue or organs and cause mortality. The aberrant traits of the cancer cells as well as the tumour growth during tumour progression stage will be further discussed in the following subchapters.

2.7 Common aberrant traits of a cancer cell

Regardless of the major differences in biological properties and diverse tissue of origin, all cancer cells share a common essential trait, i.e. growth advantage over its normal cellular neighbours. In general, cancer cells acquire its growth advantage traits in many forms and through many stages and most of these traits are actually acquired through genetic mutations, as discussed previously. The growth advantage traits found in cancer cells generally covers three main categories, i.e. excessive and uncontrolled cell growth, immortality and abnormal cell behaviour.

2.7.1 Excessive and uncontrolled cell growth

Unlike normal cell growth, cancer cell growth and division are often uncoupled from the cell differentiation and death mechanisms, leading to uncontrolled increase in tumour cell numbers. Genetic mutations, typically conversion of proto-oncogene to oncogene and deactivation of tumour suppressor genes (the gatekeeper / caretaker genes
and DNA damage sensing/reporting genes) are often the underlying cause for the cancer cell growth and proliferation (Fearon, 2000).

Proto-oncogenes are genes that produce the components of the cell signalling pathways that govern cell proliferation and survival. These genes are normally expressed in a dominant fashion and closely resemble the cancer inducing genes of the acute oncogenic retroviruses (v-ones). Examples of proto-oncogenes include genes that encode growth factors, (e.g. COLIA1-PDGFB, HST, INT-2 and KS3), receptors, (e.g. ERBB2, c-erb B1& B2, bek, flg, RET, fms and TRK), signalling molecules, (e.g. RAF, SRC, fes, RAS, mos, ABL and crk), transcription factors (e.g. MYC, fos, jun, E2F) and cell cycle regulators (e.g. CYC, CDK, BCL2 and MDM2) (Fearon, 2000; Futreal et al., 2004; Sutherland, 1999).

The tumour suppressor genes encode proteins that inhibit or retard cell proliferation for cell DNA repair or cell death purposes. Genes that come under the family of tumour suppressor genes include the caretaker genes, the gatekeeper genes and the DNA damage sensing and reporting genes. Among all, the caretaker genes encode proteins responsible for DNA damage repair (Fearon, 2000). Examples of caretaker genes include BRCA1 & 2 (which code for DNA double strand break repairers), and MDH2 and MLH1 (which code for DNA mismatch repairers) (Kohn & Bohr, 2002; Lengauer et al., 1998; Venkitaraman, 2002). Meanwhile, gatekeeper genes encode proteins responsible for cell proliferation inhibition or cell death induction in the cells that undergo non-repairable DNA damage. Examples of gatekeeper genes include p53, INK4, Rb, Chk1 & 2 and APC genes (which code for cell cycle progression inhibitors and apoptosis regulators), WT1 gene (which codes for transcription factors) and NF1 gene (which codes for ras signalling pathway regulators) (Bienz, 2002; Brown
et al., 1999; Kinzler & Volgelstein, 1997; Levine et al., 2004; Sherr & McCormick, 2002; Sherr & Roberts, 1999). DNA damage sensing and reporting genes are responsible for encoding proteins that detect DNA damage and report them to the downstream cell cycle checkpoint regulators e.g. p53 and Chk1 and 2, to coordinate cell cycle arrest, DNA repair or apoptosis (Sancar et al., 2004). Good examples of these genes include the ataxia telangiectasia mutated gene (ATM) that codes the double strand breaks detectors (Chen et al., 1999; Fernandez-Capetillo et al., 2002; Sancar et al., 2004) and ataxia telangiectasia mutated and rad3-related genes (ATR) that code for the proteins involved in detecting DNA replication blockers, single strand breaks and stalled replication forks (Abraham, 2001; Sancar et al., 2004).

In normal cells, ordered cell proliferation and differentiation occurs as a result of delicately balanced interactions between the protein products of proto-oncogenes and tumour suppressor genes. However in carcinogenesis, the gain of function mutation in proto-oncogenes and loss of function mutation in tumour suppressor genes of the cancer cells often upset such delicate balanced interactions and thus accelerate the cell proliferation. The gain of function mutations in the proto-oncogenes may cause the over production of cell growth related proteins (e.g. excess production of the transcription factor Myc protein due to gene amplification of the MYC gene, in the case of Burkitt’s lymphoma) (Schwarb, 1988) or the production of protein that exhibit enhanced cell growth and proliferation activities (e.g. the Trk fusion protein made by the TRK oncogene in the case of the thyroid cancer) (Butti et al., 1995). These mutated proto-oncogenes (termed oncogenes) are generally expressed in a dominant fashion (i.e. only mutation in one of the two copies of the proto-oncogenes is required to initiate the neoplastic change) (Evans, 2002).
Meanwhile, the loss of function mutation of the tumour suppressor genes may lead to defective or failure in DNA damage detection, reporting and repair, cell cycle arrest initiation and apoptosis induction. As a result, the cell is allowed to proliferate without any safeguarding mechanism against any lethal mutations and the acquired mutations are allowed to be passed down to the daughter generation, causing accumulation of mutations in the cell genome and conversion of the cell to cancerous cell. However, such devastating events will only occur if the cells lose both copies of their normal tumour suppressor genes, according to the Knudson’s two hit model (Knudson, 1971). As cancer cells proliferate, subsequent accumulation of mutations in a number of different oncogenes and tumour suppressor genes may happen due to intense DNA replication activity. Regardless of the type, the end results of the occurrence of these mutations would always be disregulated expression and function of the corresponding gene product, leading to further cellular proliferation that is normally associated with increase in tumour size, genomic disorganisation and malignancy.

2.7.2 Immortality

In normal eukaryote cells, the shortening of the DNA protective telomeric sequences (due to the inefficiency of the DNA polymerase in copying the end portion of the chromosomal DNA strand) after every DNA replication has eventually led to the loss of informative DNA sequences and chromosome instability in the cells, and at the end cell senescence and apoptosis (Bedi & Kastan, 2000; Blasco, 2002). However in cancer cells, the threat of telomeric sequence shortening is circumvented by the persistent production of the enzyme telomerase that is capable of replenishing the lost telomeric sequences to the chromosomal DNA (Bedi & Kastan, 2000; Blasco, 2002). As long as the cancer cells do not meet up with any fatal events or mutations, they are
considered to be immortal, that is, able to live and replicate for an indefinite period. Immortality is an important feature that allows the cancer cells to outgrow the mortal cells of normal tissue surrounding them. As the normal cells die naturally due to telomere shortening, the cancer cells divide and take over interstitial space previously occupied by the dead cells, thus leading to dominance of the cancer cell colony in the tissue region.

### 2.7.3 Abnormal cell traits

Apart from uncontrolled cell proliferation and immortality, cancer cells also possess a number of abnormal cell traits that enable them to outgrow the normal cells around them. These abnormal cell traits are often acquired by the cancer cells through a series of gene mutations that occur along the cells’ indefinite and uncontrolled DNA replication and cell proliferation. At times, continual exposure of cancer cells to external stimulations, for example free radicals generated by the immune cells that are attacking the cancer cells, chemical carcinogens or even chemotherapeutic agents may induce further gene mutations that offer more abnormal traits to the cancer cells and render the cancer cells more adaptable to the surrounding environment. The abnormal traits that are commonly acquired by the cancer cells include:

i. **Self stimulation for accelerated and persistent cell proliferation** through production of hyperactive version / excessive quantities of growth factors (e.g. platelet-derive growth factor, PDGF and epidermal growth factor, EGF) or permanently activated growth factor receptors (Bedi & Kastan, 2000; Ruddon, 2000).
ii. **Insensitivity towards the influence of the antigrowth signals** brought by external antigrowth factor such as TGF-β, by producing inactivated cellular components involved in the TGF-β antigrowth signalling (e.g. mutated smad or Rb protein) (Massague *et al.*, 2000) or by inactivating the Rb proteins via viral proteins (e.g. E7 protein by HPV) (zur Hausen, 2002).

iii. **Enhanced invasiveness** to the surrounding tissue via the production and release of protease (Fidler, 2000; Friedl & Wolf, 2003), that degrade the surrounding protein based tissue structure (e.g. cathepsin B, degrades collagens, fibronectins, and lamlin) (Buck *et al.*, 1992) or serve as activators (e.g. plasminogen activators) to activate the other protease precursors found in the surrounding regions (e.g. activation of plasminogen to plasmin that is capable of degrading the basal lamina and extracellular matrix and activating the metalloproteinases), so as to create more space for cancer growth and migration (Fidler, 2000; Friedl & Wolf, 2003).

iv. **Ability to metastasize**, the cancer cells may obtain this trait upon the acquisition of a few other abnormal traits, such as, the reduced adhesiveness among the cancer cells (due to the lacking of cell adhesion molecule E-cadherin) (Hanahan & Weinberg, 2000), the anchorage independency growth of the cancer cells (Frisch & Screaton, 2001; Ruoslahti & Reed, 1994; Ruddon, 2000) and the ability to induce angiogenesis around the cancer cell colony through consistent production and spillage of angiogenic factors (e.g. vascular endothelial growth factors, VEGF and fibroblast growth factor, FGF) into the surrounding tissue region (Carmeliet, 2000; Carmeliet & Jain, 2000).
v. **Multi-drug resistance**, through the increased production of the p-glycoprotein (P-gp, code by MDR-1 gene), a membrane bound multi-drug transporter that is capable of pumping various cytotoxic substances out of a cell (Dean *et al.*, 2005; Gottesman, 2002). Significant upregulation of the P-gp production in the cancer cells may occur in response to the chemotherapeutic drug induced cytotoxic stress during chemotherapy. Comparatively, such upregulation in the production of the P-gp may not occur in time in normal cells under similar cytotoxic stress as normal cells often enter into apoptosis before their MDR-1 genes are upregulated sufficiently to overcome the toxicity (Dean *et al.*, 2005; Gottesman, 2002).

vi. **Immuno-evasiveness**, either through generation of cancer cells clones with reduced or no expression of tumour specific antigens, or expression of tumour antigens unrecognized by immune cells e.g. macrophages and dendritic cells, B and T lymphocytes, or simply by rapidly proliferation to overwhelm the anti-tumour efforts of the immune cells such as the NK cells (Hanahan & Weinberg, 2000). Furthermore, the tumour may also surround itself with a dense layer of connective tissue to shield itself from an immune cell’s attack.

With the aforementioned growth advantage traits, cancer cells stand a higher chance to dominate over normal cells in competition for survival, growth space and nutritional supply, no matter at the primary tumour growth site or the tumour metastasis sites. Eventually, the growth and function of the normal cells adjacent to the tumour tissue will be severely impaired. Death of normal tissue and organ will soon occur and in the end, death of the host will take place.
2.8 Treatment of cancer

Currently, the methods employed in cancer treatment include surgery, radiotherapy, chemotherapy, hormonal therapy and immunotherapy.

2.8.1 Surgery

Surgery involves surgical removal of tumour masses and the surrounding tissues from the growth site. Besides using conventional metal scalpel and surgical instruments to remove the tumour mass, other means of more advanced techniques, for example laser surgery, electrosurgery, cryosurgery and high-intensity focused ultrasound (HIFU) are employed as well (Allen-Mersh, 1996; Niederhuber, 2000). Regardless of the techniques, surgical treatment of cancer bears a few shortcomings. Firstly, the treatment can only bring cure to a cancer patient if the cancer is discovered in its early stage of development, i.e. before cancer metastasis begins (Niederhuber, 2000). In advanced and metastatic cancer cases, surgery may only serve to control the disease and to improve the quality of life of the patient. Secondly, the surgeon may find difficulties in removing the entire tumour mass from the growth site if the tumour is deeply seated in delicate organs, e.g. in some cases of brain tumour.

2.8.2 Radiotherapy

Radiotherapy refers to treatment methods that inflict fatal DNA damage in the cancer tissue using high energy ionizing radiations (Bernier et al., 2004; McBride et al., 2002). The cancer cells are more susceptible to radiation damage and radiation induced cell death compared to normal cell due to their defective DNA repair machinery.
(Siddik, 2002). Upon DNA damage, cancer cells may enter into p53 mediated apoptotic death or mitotic death (Bernier et al., 2004; McBride et al., 2002). In clinical practice, the anticancer radiotherapy methods employed include the **external beam radiotherapy** (EBRT) that uses megavoltage X-rays, γ ray (from $^{60}$Co, $^{192}$Ir, $^{137}$Cs or $^{226}$Rd), electron and proton beams for cancer annihilation (Hartford et al., 1999; Williams & Thwaites, 2000), **brachytherapy** that uses β particle or γ ray emitted from a sealed radioactive source (e.g. $^{90}$Sr plaque and $^{125}$I seed) deployed inside or next to the cancer growth area to treat cancer, and the **unsealed source radiotherapy** that treats cancers via the administration of radionuclide alone (e.g. $^{131}$I and $^{89}$Sr) or radionuclide conjugated to soluble biomolecules (e.g. $^{131}$I-metaiodobenzylguanidine) into the body by injection or ingestion (Volkert & Hoffman, 1999). At present, radiotherapy is used for curative, adjuvant or palliative cancer treatment. In general, the therapy does not inflict pain and causes minimal or no side effects when the radiation is employed at low-dose (e.g. in palliative treatments to bony metastases). However, curative or adjuvant cancer treatments that involve the use of higher doses may cause side effects such as burns (on the surface exposed to the radiation), oedema, infertility, fibrosis, hair loss, generalized fatigue, etc. Similar to surgery, radiotherapy may only work well in treating cancer at its early stages of development, i.e. prior to metastasis and has a limited role in the treatment of widely disseminated cancers (NCCP, 2002).

### 2.8.3 Chemotherapy

Unlike surgery and radiotherapy, chemotherapy employs cytotoxic drugs in destroying tumour tissue, thus effecting cures in certain cancers even when the disease is disseminated (e.g. in Hodgkin’s disease and high grade non-Hodgkin lymphomas, germ cell tumours, leukaemias and limited stage small cell lung cancer) (NCCP, 2002).
The anticancer drugs employed in the therapy are normally administered intravenously to the patient. However, other means of drug administration such as oral, transdermal intra-tumoural and intra-thecal drug administration are also used. The chemotherapeutic agents employed in cancer treatment include antimetabolites, alkylating and platinating agents, antibiotics and plant derived-drugs.

The antimetabolites are molecules that structurally resemble or partly resemble to some of the key substrates (metabolites) required for biochemical reactions that are essential for cancer cell survival, such as DNA replication, RNA synthesis and the cellular enzyme reactions that are related to these activities (e.g. the pyrimidine and purine nucleotide synthesis and phosphorylation, etc.) (Grem & Keith, 2002). Upon entering the cancer cell, the antimetabolites may compete with the actual metabolites for enzyme binding sites, block the enzyme active sites (temporary or permanently) to inactivate the enzymes and disrupt the DNA / RNA synthesis and DNA repair processes through mechanisms such as masked chain termination and DNA / RNA nucleotide incorporation inhibition, thus interfering with the normal progression of the aforementioned biochemical reactions and subsequently bringing death to the cancer cells. Examples of the antimetabolites include pyrimidine analogs (e.g. floxuridine, fluorouracil, cytarabine, capecitabine, gemcitabine and troxacitabine), purine analogs (e.g. cladribine, clofarabine, fludarabine, pentostatin, mercaptopurine, thioguanine and azathioprine) and folic acid antagonist (e.g. aminopterin, methotrexate, pemetrexed, raltitrexed) (Grem & Keith, 2002; DBD, 2006a).

The alkylating agents are chemicals that can attach an alkyl group to the cellular DNA, typically on the guanine nucleobases in DNA double-helix strands. By doing so, alkylating agents form cross-linkages between the DNA double helix (dialkylation
agents) and Limpet attachment (monoalkylating agents) on the single DNA strand, resulting inhibition in DNA double helix separation and DNA transcription. Chronic exposure of the normal cells to these agents may lead to genetic mutation and cancer development (subchapter 2.3.1). However, in cancer treatment, high dose exposure of cancer cells to these agents may cause fatal DNA damage as the cancer cells often have defective DNA repair mechanisms (Siddik, 2002). Examples of alkylating agents include the nitrogen mustards (chlorambucil, chlormethine, cyclophosphamide, ifosfamide, melphalan), nitrosoureas (carmustine, fotemustine, lomustine, streptozocin), temozolomide, mechlorethamine, busulfan, procarbazine, dacarbazine, thioTEPA and uramustine (DBD, 2006b). Meanwhile, the platinating agents kill cancer cells by forming crosslinks between the DNA double helix (Siddik, 2002). These agents contain platinum atom(s) in their molecular structure. The examples of the platinating agents include carboplatin, cisplatin and oxaliplatin (DBD, 2006c).

The antibiotics and plant derived alkaloids are organic molecules derived from living organisms (e.g. bacteria and fungi) or plants. These molecules kill cancer cells through various mechanisms, whereby many of these mechanisms disrupt the cells’ DNA replication / repair and mitotic activities (Sparreboom et al., 2002). Examples of antibiotic anticancer agents include mitomycin (DNA crosslinking agent), bleomycin (DNA strand break inducer) and anthracyclines (doxorubicin, epirubicin, idarubicin mitoxantrone, valrubicin and danaurubicin, topoisomerase II inhibitors) (DBD, 2006d). Examples of plant derived anticancer alkaloids include the spindle poisons taxane (docetaxel, paclitaxel) and vinca alkaloids (vinblastine, vincristine, vindesine, vinorelbine), the topoisomerase I inhibitors camptothecin, topotecan and irinotecan, and the topoisomerase II inhibitors etoposide, and teniposide (DBD, 2006e).
In general, chemotherapy is valuable for curative, adjuvant, palliation cancer treatments for cancer of all stages (NCCP, 2002). However, the wide and unrestricted dissemination of small molecular size anticancer drugs in the body upon drug administration often reduces the bioavailability of the drug to the tumour and at the same time causes non-specific cytotoxicity to normal healthy tissue. The anticancer drugs’ non-specific cytotoxicity to body tissue often accounts for the various adverse effects occurring in patients (e.g. bone marrow suppression, nausea and vomiting, stomatitis, abdominal pain, constipation, diarrhoea, hair loss, etc.) during intensive chemotherapy (OCC, 2003). Furthermore, the use and the efficacy of a number of the chemotherapeutic drugs (for example, paclitaxel, docetaxel, methotrexate, daunorubicin, epirubicin, melphalan, fluorouracil, and others) are often limited due to their water insolubility or poor water solubility characteristics. Although surfactants are used at times in the parenteral administration of these drugs to increase the drugs’ miscibility in the blood, these surfactants are in turn found to carry a certain level of toxicity and can become the treatment dose limiting factor (e.g. the use of Cremophor EL in the case of paclitaxel) (Li et al., 2002; Weiss et al., 1990). In spite of its shortcomings, chemotherapy still remains as a potential option in cancer treatment. In view of this, modification of the currently available cytotoxic drugs’ tissue distribution patterns and tumour targeting properties would be essential to overcome the shortcomings and improve the effectiveness of the chemotherapy.

2.8.4 Hormonal therapy

Hormones are chemical transmitters that are commonly used by the body to convey various biological signals (e.g. cell growth and differentiation, protein synthesis, proliferation halt etc.). They can also be used for cancer treatment. Examples of
hormones used in cancer treatment include tamoxifen (estrogen analogs for treating estrogen receptor positive (ER+) breast cancers, Jordan, 2006), androgen analogue (leuprolide) and androgen receptor blockers (e.g. flutamide and bicalutamide) (Damber, 2005). Compared to cytotoxic drugs, treatment by hormone brings lesser adverse effects as normal cells are generally not affected. However, the use of hormones is often limited to the treatment of cancers that exhibit hormone dependent growth characteristics or are sensitive to hormonal changes.

### 2.8.5 Immunotherapy

Monoclonal antibodies are a more recent class of compound employed in treatment of cancers. In essence, monoclonal antibodies synthesized by immortalized plasma cells (through hybridoma technology, plasma cells were previously exposed to the tumour antigen) are capable of recognizing and binding specifically to fixed antigens that are only found on the surface of cancer cells (Blattman & Greenberg, 2004). Binding of the antibody to the tumour cells may trigger instant cancer cell death (by binding and blocking the tumour cell growth receptors) or they may draw immune attack onto the cancer cells by acting as antigenic beacons to the body immune system. At times, antibodies are used as delivery vehicles to deliver cytotoxic drugs and radionuclides to the vicinity of the tumour by allowing these substances to bind to them and then to the cancer cells (Lake & Robinson, 2005). Examples of antibodies that are employed clinically in cancer treatments include Rituxan, Zevalin and Bexxar for treatment of non-Hodgkin’s lymphoma, Avastin for leukemia treatment and Herceptin for breast cancer treatment (Waldmann & Thomas, 2003). Although effective in recognizing cancer cells with reduced adverse effects, antibody therapy often encounters problems such as loss of the antibody’s effectiveness due to recognition and
inactivation of the protein based antibodies by the body’s immune system before reaching the tumour site, as well as the potential change of the antigen expression on the cancer cell surface, as the cancer cells continue to proliferate and mutate. Also, the antibody may falsely interact with normal cells if the tumour antigen it targets is expressed on the normal cells (Blattman & Greenberg, 2004). Additionally, the high cost incurred during monoclonal antibody production also limits the use of the therapy at present stage.

### 2.9 Improving chemotherapy through anticancer drug delivery strategies

The role of chemotherapy in cancer treatment is irreplaceable and modification of currently available cytotoxic drugs’ tissue distribution patterns and tumour targeting properties may overcome the shortcomings and improve the effectiveness of the chemotherapy. Among the potential ways of doing this is to deliver the drugs to the tumour tissues using specific drug carriers, for example, liposomes, niosomes, nanoparticles, microparticles, polymeric micelles, immunoconjugates and polymer-drug conjugates (Allen & Cullis, 2004, Duncan 2003; Torchillin, 2005).

#### 2.9.1 Liposome

Discovered in the mid 1960s (Bangham et al., 1965), liposome is a drug delivery vehicle that can potentially protect the drugs from blood degradation and restrict the random diffusion of drugs in the body (Minko et al., 2006). Liposome is a 70-200 nm sized amphiphilic spherical phospholipid bilayer vesicle (Figure 2.9.1) that is capable of encapsulating aqueous soluble drugs within its central aqueous compartment or lipid
soluble drugs within its lipid bilayer membrane (Minko et al., 2006). Drugs encapsulated by the liposome can readily pass through the cell membrane upon fusion of the liposome lipid bilayer with the cell membrane. Liposomes can be constructed using naturally-derived phospholipids with mixed lipid chains (like egg phosphatidylethanolamine), or of pure surfactant components such as DOPE (dioleolylphosphatidylethanolamine) (Minko et al., 2006). The application of liposomes to deliver the anticancer drugs was initially hampered by the liposome’s short blood circulation time due to the rapid clearance of the liposomes by the liver and spleen macrophages (Gregoriadis & Ryman, 1972). However, the potential of liposomes in anticancer drug delivery was again highlighted following the development of the stealth liposome, that is, liposomes coated with surface ligands such as monosialoganglioside (Gabizon & Papahadjopoulos, 1992) and polyoxyethylene (Blume & Cevc, 1990; Maruyama et al., 1991) that can reduce the liposome opsonisation by plasma proteins and thus prevent liposome recognition by the liver and spleen macrophages (Allen, 1994; Senior et al., 1991), and also the development of a few other new non-stealth liposome species with extended blood circulation time, for example, liposomes made of glucoronic acid lipids (Namba et al., 1992), cholesterol (Kirby & Gregoriadis, 1983), distearoyl phosphatidylecholine (Forssen et al., 1992) or polyvinyl-pyrrolidone polyacrylamide lipids (Torchilin et al., 1994). The new generation of stealth and non stealth liposomes was proposed to passively target solid tumours by extravasation into the tumour interstitium through the disorganised tumour vasculature (Gabizon et al., 1990; Forssen et al., 1992; Huang et al., 1992; Wu et al., 1993). The examples of the stealth and non stealth liposome-anticancer drug constructs are respectively the liposomal doxorubicin (licensed as Caelyx) (Gabizon et al., 1993, 1996; Winterhalter et al., 1997) and the distearoyl phosphatidylecholine (DSPC) based liposomal daunorubicin (licensed as Daunoxome) (Gilead, 2006), both for the treatment of Kaposi’s sarcoma.
(Bergin et al., 1995; Lasic & Papahadjopoulos, 1995) and ovarian cancer (FDA, 2005). Currently, the development of the more advanced liposome species, for example, the thermo sensitive liposomes (Maruyama et al., 1993), immunoliposomes (Ahmad et al., 1993; Huwyler et al., 1997), organ specific targeting liposomes (Abra, 1984; Litzinger & Huang, 1992), anti-multidrug resistance liposome (Krishna & Mayer, 1997) and ligand bearing liposome (Forssen & Willis, 1998) are in progress and more liposomal formulations of the anticancer drugs, for example, paclitaxel. (Sharma et al., 1993), 5-fluorouracil (Doi et al., 1994) and vincristine (Mayer et al., 1995; Tokudome et al., 1996) are in preclinical development. Despite this rapid development, liposome constructs have yet to demonstrate their superior efficiency in terms of \textit{in vivo} solid-tumour targeting (Duncan, 1999). Furthermore, neurotoxicity that can be potentially brought by a certain class of liposome may limit the use of these drug delivery systems. (Adams et al., 1977)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{liposome.png}
\caption{Liposome construct (size: 70-200 nm)}
\end{figure}

(Figure adapted from the website: \url{http://www.hbprotocols.com/ketocream/qa.html}, access date:4/6/2007, with modifications)
2.9.2 Niosome

Niosomes or non-phospholipid liposomes are closed bilayer vesicles formed from the hydration of a mixture of cholesterol and amphiphilic non-ionic surfactants such as polyoxyethylene, triglycerol, palmitoyl muramic acid, hexadecyl poly-5-oxyethylene ether, octadecyl poly-5-oxyethylene ether, hexadecyl diglycerol ether, sorbitan monopalmitate and sorbitan monostearate. (Uchegbu & Duncan, 1997; Uchegbu & Vyas, 1998). These vesicles were found to accumulate within tumours in a manner similar to liposomes (Rogerson et al., 1988; Uchegbu et al., 1995). Niosomes have been used for the encapsulation and tumour delivery of anticancer drugs such as doxorubicin (Rogerson et al., 1988; Uchegbu et al., 1995), vincristine (Parthasarathi et al., 1994) bleomycin (Naresh et al., 1996) and anticancer polymer drug conjugate PK1 (Uchegbu & Duncan, 1997). Although increases in drug (doxorubicin and methotrexate) delivery to the tumour and tumouricidal activity were seen in the study using triglycerol and sorbitan monostearate niosomes (Kerr et al., 1988; Rogerson et al., 1988), no significant improvement in antitumour activity was observed in the study using polyoxyethylene-doxorubicin niosomes (Uchegbu et al., 1995). Certain noisome constructs, for example, palmitoyl muramic acid niosomes and polyoxyethylene niosomes can be taken up by the spleen and liver rapidly (Uchegbu et al., 1995; Uchegbu, 1998). Hence, the use of these niosomes for anticancer drug delivery may lead to premature blood clearance of the drug and increase the risks of liver and spleen toxicity.
2.9.3 Microparticles and nanoparticles

Microparticles (particle size >0.5 μm) and nanoparticles (particle size: 30-500 nm) are solid polymer matrix cored particles synthesized for drug targeting and drug controlled release purposes. These particles are made of polymer materials such as non-biodegradable polymer polystyrene (Illum et al., 1983); biodegradable polymers poly lactic acid, poly glycolic acid, poly ß-hydroxybutyrate and fibrin (Brannonpeppas, 1995; Couvreur et al. 1995; Senderoff et al., 1991); and the bioerodible polymers alkyl cyanoacrylates (Couvreur et al. 1995). Drug loading into these particles is performed simultaneously through physical entrapment during particle formation (Cavalli et al., 1993; Thompson et al., 1997). Between the two particle types, drug loaded nanoparticles are injected intravenously for passive tumour drug targeting purposes. Like the liposomes and niosomes, nanoparticles can be cleared from the blood circulation by the liver and spleen (Illum et al., 1983; Verdun et al., 1990). To circumvent such undesirable nanoparticle blood clearance, stealth nanoparticles are
developed, either by coating the ordinary particles with soluble polyoxyethylene (Leroux et al., 1996) or by constructing the nanoparticles using block polyoxyethylene copolymers or dialkyl polyoxyethenenes and phospholipids (Bazile et al., 1993; Gref et al., 1994; Hodoshima et al., 1997; Verrecchia et al., 1995). Stealth nanoparticles were reported to be capable of increasing the drug accumulation in tumour and enhanced the drugs’ tumouricidal activity in mice (Hodoshima et al., 1997; Leroux et al., 1996). However, premature release of drugs may occur before the particles arrive at the tumour site since the drugs are housed in these particles via physical entrapment. Examples of stealth nanoparticles include of paclitaxel poly(D,L-lactide)/methoxy poly(ethylene glycol)-poly(D,L-lactide) nanoparticles (Dong & Feng, 2006) and adriamycin polyethylene glycol-lipid nanoparticles (Hodoshima et al., 1997). Meanwhile, microparticles are injected intraperitoneally, intramuscularly, subcutaneously or directly to the target organ or tumour, as a sustained release depot for the drug (Codde et al., 1993; Ike et al., 1991). Drug is gradually released by diffusion from the particles or upon particle erosion (Moritera et al., 1991; Mehta et al., 1994; Ruiz et al., 1991). Although potential exists in treating local malignancies, drugs loaded microparticles are not used to treat metastatic cancer as these particles are not suitable for intravenous administration due to their relative large size (which makes them potential emboli in the blood). Examples of drug loaded microparticles include doxorubicin ion exchange resin microparticles (Codde et al., 1993), 5-fluorouracil Poly(lactic acid-co-glycolic acid) microparticles (Menei et al., 1993) doxorubicin albumin microparticles (Willmott et al., 1987), mitozantrone chitosan microparticles (Jameela et al., 1996) and doxorubicin polylactic acid microparticles (Ike et al., 1991).
2.9.4 Polymeric micelles

Amphiphilic block copolymers such as poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) copolymers and poly(ester) hydrophobic-block-poly(L-amino acid) copolymers are capable of self-assembling themselves into polymeric micelle (Figure 2.9.4), a colloidal structure capable of housing drug molecules within its core or its polymer network (Batrakova et al., 1996; Kabanov et al., 1992; Yokayama et al., 1990). Polymeric micelles were found to have good blood miscibility and stability and may circulate in the blood circulatory system for prolonged periods of time (Kwon et al., 1993 & 1994). As such, it may deliver more drugs to tumour tissue when compared with administration of the drug in solution (Kwon et al., 1994) Since this is a rather dynamic system where the exchange of micelle structural components between the micelle structure and its surrounding environment occurs continuously (Florence & Attwood, 1998), premature release of drugs out of the micelle structure is possible, especially for drugs that are housed in the micelles via physical entrapment. Examples of polymeric micelles include epirubicin.
and doxorubicin loaded Pluronic® (poly(ethylene oxide)-block-poly(propylene oxide)-
block-poly(ethylene oxide) copolymers) micelles (Batrakova et al., 1996) and the
doxorubicin loaded polyoxyethylene block polyaspartic acid micelles (Yokayama et al.,
1990).

Figure 2.9.4 Simple polymeric micelle

(figure adapted from the website:
http://www.uweb. engr.washington. edu/research/tutorials/drugdelivery.html access date: 4/6/2007, with
modifications)

2.9.5 Immunoconjugate

Meanwhile, immunoconjugate drug delivery strategy utilizes monoclonal
antibodies (MAbs) that target antigens on the tumour cell surface (for example, folic
acid receptor, LDL receptors, peptide receptors, proteins and lipid components) as the
carriers to deliver the drug molecules to the tumour tissue (Chung & Wasan, 2004;
Jendrossek & Handrick, 2003; Lu & Low, 2002; Minko et al., 2004; Nielsen et al.,
2002). The drugs are normally conjugated chemically to the MAbs for example, at the
Fc-portion of the antibody. Once the drug laden antibodies bind to the antigens on the
tumour cell surface, the drugs linked to the antibody are released by means of drug-
antibody linkage hydrolysis or antibody endocytosis and degradation by the cells.
Examples of anticancer immunoconjugates include MAb-calicheamicin (Mylotarg®,

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for acute CD33 +ve myeloid leukaemia treatment) (Giles, 2002), MAb-bombsein (for treatment of the small cell carcinoma of the lung) (Chen et al., 1995) and MAb-N2'-deacetyl-N2'-(3-mercapto-1-oxopropyl) (Cantuzumab Mertansine, for pancreatic, biliary, colorectal, gastric, uterine, bladder and non-small cell lung cancers treatments) (Tolcher et al., 2003). Although being heralded previously as the ‘Magic Bullets’ that would seek out and selectively destroy tumour tissue, immunoconjugates have so far proved disappointing in the context of in vivo solid-tumour targeting (Duncan, 1999). Furthermore, the low drug loading capacity of the immunoconjugates and the high risk of inactivation of the immunoconjugates by the body immune system (immune response mounted against the MAb drug carrier) often hamper the use of these drug delivery systems.

Figure 2.9.5 Immunoconjugates

(figure adopted from the website: http://www.biotech.sunysb.edu/educWork/ibrp/iojim.html, access date: 4/6/2007, with modification)
2.10 Drug delivery through the polymer drug conjugate strategy

Evolving in parallel, drug tumour targeting through the polymer-drug conjugate based drug delivery strategy has drawn a considerable amount of interest and attention of the research community in the past 2 decades. The concept of polymer - anticancer drug conjugation was first envisioned by Helmut Ringsdorf in 1975 (Ringsdorf, 1975). Under the polymer - drug conjugate strategy, the cytotoxic drugs are bound chemically to the water soluble biocompatible polymers (e.g. hydroxypropylmethacrylamide (HPMA) polymer, albumins, polyamino acids, polyethylene glycol, dextran, etc.) directly or using linkers (e.g. aldehyde / Schiff base linkers, sulphhydryl linkers, acid-labile linkers, \textit{cis}-Aconityl linkers, hydrazone linkers, enzymatically degradable peptide linkers, etc.) (Garnett, 2001). The water soluble polymers employed in the polymer drug conjugate (PDC) development may come in various structures and architectures. This include the classical random coiled linear polymers (Duncan \textit{et al}, 1996), branched polymers (Stiriba \textit{et al}, 1998), multivalent polymers (Mamman \textit{et al}, 1998), block polymers (Pechar \textit{et al}, 2000), graft polymers (Ferruti \textit{et al}, 1998), dendrimers (Frechet, 1996; Tomallia \textit{et al}, 1985), dendronized polymers (Malenfant & Frechet, 2001), star polymers (Mishra & Kobayashi, 1999), and others. The general scheme of a classical linear polymer-anticancer drug conjugate (PDC) can be found in Figure 2.10.

![Figure 2.10 Linear polymer-anticancer drug conjugate (PDC)](image-url)
Compared to physical entrapment / encapsulation of chemotherapeutic drugs in drug carriers such as liposomes, niosomes, nanoparticles, microparticles or polymeric micelles, chemical conjugation of chemotherapeutic drugs to water soluble polymers is a more attractive approach in reducing systemic toxicity, prolonging \textit{in vivo} half-life, enhancing tumour targeting properties and improving the therapeutic index of the drugs (Duncan & Spreafico, 1994). Once bound to the polymer, chemotherapeutic drugs will be stabilized or inactivated temporarily until they are released again (through cleavage of the chemical linkage in between the drug and the polymer or the peptide linker that link the drug to the polymer by means of hydrolysis or by the action of cellular enzymes, e.g. cathepsin) (Duncan & Spreafico, 1994). The molecular weight of polymeric drug carriers employed in the polymer drug conjugation is carefully controlled at a level that is larger than 30 kDa so that PDCs do not readily diffuse through the gaps and fenestrations found along the capillaries of the normal tissue (Duncan & Spreafico, 1994; Maeda & Matsumura, 1989; Reynolds, 1995). Through this, random diffusion of the chemotherapeutic drug loaded polymer drug conjugates (PDCs) into the normal tissue is minimized. For the PDCs that are made of biodegradable polymers, the PDC size can be increased to over 45 kDa to minimize rapid renal clearance of PDCs post intravenous administration, and thus increase the plasma half life of PDCs and their drug payload (Seymour \textit{et al}., 1987). All these may spare the normal tissue from irrelevant drug-mediated toxicity and at the same time increase the drug concentration available in the blood (Maeda & Matsumura, 1989; Reynolds, 1995).

Meanwhile, by binding the drugs to the polymers, drugs administered into the blood circulation are largely protected by the polymeric carrier’s structure from premature degradation by plasma enzymes (e.g. gemcitabine, cytosine arabinoside and
5-azacytidine degradations by plasma cytidine deaminases) (Drake et al., 1980; Immordino et al., 2004) and random hydrolysis. The hydrophilic characteristics of the polymers may also give the PDCs a form of steric barrier against interactions with plasma proteins, such as opsonins and lipoproteins (Storm et al., 1995). As such, PDCs may evade capture by the mononuclear phagocyte system (MPS) (Storm et al., 1995). Together with the extensive stability (delayed plasma degradation) of the polymeric carrier in the blood, all the abovementioned factors may result in prolonged circulation time of the PDCs in the blood compartment, giving the PDC more time for tumour targeting (Peracchia et al., 1999; Storm et al., 1995).

While travelling along the blood stream, the PDCs locate the solid tumour site through passive or active targeting mechanism, depending on their structural design. Between the two, passive targeting mechanism is the general mechanism adopted by most of the PDCs developed presently. This targeting mechanism functions mainly based on the enhanced permeability - retention (EPR) effect, a phenomenon derived from the unique anatomical features of the tumour vasculature.

The EPR effect refers to the phenomenon whereby the macromolecules in the blood stream (e.g. albumins, hormones, lipoproteins, etc.) are found trapped in the tumour interstitium as these molecules exit from the blood capillary. The EPR effect is one of the measures that the tumour tissue employs in acquiring more proteins, nutrients, hormones and growth factors for themselves from the blood stream (Maeda & Matsumura, 1989, Reynolds, 1995). From previous studies, it was found that malignant tumours often possess dense but leaky vascular networks with multiple gaps and fenestrations (600 – 800 nm wide) on the capillary walls (Allen & Cullis, 2004; Dvorak et al., 1988; Maeda & Matsumura, 1989; Reynolds, 1995). At the same time, the tumour
vascular endothelium also exhibit elevated macromolecule transport (transcytosis) activity through their vesiculo-vacuolar organelle systems (Kohn et al., 1992; Maeda et al., 2000; Matsumura & Maeda, 1986). Such distorted vascular features have conferred the tumour vasculature greater permeability to macromolecules compared to the normal tissue vasculature, even in the presence of high tumour interstitial back pressure (Duncan, 1999; Fidler, 1987; Kohn et al., 1992; Maeda & Matsumura, 1989), thus encouraging the extravasation of macromolecules from the blood stream into the tumour interstitium. At the same time, tumours often lack a lymphatic vasculature to remove large molecules that leak into the tumour tissue (Maeda & Matsumura, 1989; Reynolds, 1995). In such instances, entrapment of the macromolecules in the tumour interstitium is often the end result.

Upon discovery of the EPR effect, the phenomenon has been exploited extensively by the research community in the development of self targeted macromolecular size anticancer PDCs. Under the influence of the EPR effect, PDCs that persistently travel in the blood stream (as they are too large to pass through the normal vessel walls unless they are trapped by the reticuloendothelial system in organs such as liver, bone marrow and spleen) may selectively extravasate out of the hyperpermeable tumour blood vessels together with the other macromolecules in the blood, enter into the solid tumour interstitium and be retained for a long period of time.

In the tumour interstitium, the PDCs will either go through gradual extracellular degradation (hydrolysis or enzyme degradation, e.g. by cathepsins and proteases) to release the anticancer drugs or they might eventually be taken up by tumour cells via endocytosis and release the drug from the conjugate upon PDC degradation by cellular enzyme or hydrolysis (Garnett, 2001). Both mechanisms may result in a more specific
tumour accumulation of the active therapeutic drug and hence annihilation of the tumour cells (Duncan & Spreafico, 1994). Additionally, the infiltrated PDCs may act as drug depots for sustained release, producing prolonged drug exposure to tumour cells. The use of water soluble polymers will also help solubilize some otherwise insoluble drugs (e.g. conjugation of paclitaxel to poly-glutamic acid) and ease the administration of these drugs to patients (Li et al., 2002).

The PDCs that function under passive targeting mechanism are normally constructed using linear biocompatible polymers strands that are formed by a single type of monomer, a mixture of a few types of monomers or alternate stretches of 2 different types of polymers (block copolymer). At times, branched polymers, dendronized polymers, dendrimers are also employed in the PDC construction to suite specific needs, e.g. to increase drug payload. Some examples of passive targeting PDCs can be found in Table 2.11.

Despite the provision of more specific tumour targeting and lesser toxicity to the normal tissue, PDCs do have their shortcomings. For example, the PDCs may enter the interstitium of the spleen, liver and bone marrow, as these organs possess blood vessels with fenestrated or discontinuous endothelium (Garnett, 2001). However, clearance of PDCs from these organs is possible as the EPR effect would not occur here.

The idea of active PDC targeting was recently proposed in the effort to enhance PDC tumour targeting ability. In the active PDC targeting approach, targeting molecules such as monoclonal antibodies or ligands against tumour related receptors are linked onto the developed passive targeting PDCs. Upon entry to tumour interstitium, these modified PDCs may selectively bind to cancer cells with the help of targeting
molecules (i.e. through antibody - antigen or ligand - receptor binding mechanisms). Once bound on the tumour cell surface, the targeting molecules, together with the PDCs will be endocytosed by the cancer cells through clathrin-mediated endocytosis (Mellman, 1996). Degradation (enzyme based or hydrolysis) of the PDCs in the cancer cell cytoplasm may release the anticancer drugs into the cell cytoplasm, and thus kill the cancer cell. Examples of PDCs in this class include the biotinylated poly-ethylene glycol - camptothecin that interacts with the sodium dependent multivitamin transporter (SMVT) of tumour cells (Minko et al., 2002), antibody labelled HPMA-doxorubicin and antibody labelled multiblock poly(ethylene glycol)-doxorubicin (Pechar et al., 2003).

While having an improved targeting profile, the PDCs functioning under active targeting mechanisms do have one disadvantage, i.e. they are often at risk of being discovered, recognized and neutralized by the body’s immune system as the protein targeting molecules attached to them can be potentially antigenic, typically in the antibody labelled PDCs. Such disadvantage often causes the PDCs of this class to lose their effectiveness after their extensive use in vivo.

### 2.11 Development of the anticancer polymer drug conjugates

In spite its shortcomings (no matter in the passive or active tumour targeting PDCs), the polymer - anticancer drug conjugation has been recognized and accepted by the research and clinical communities as one of the potential approaches in generating new generations of chemotherapeutic agents with improved tumour targeting profile and less adverse effects. Preliminary research on copolymer-based anticancer conjugates has progressed rapidly and many synthetic and natural polymers have been examined for their ability to enhance tumour-specific drug delivery. A variety of PDC based anticancer drug derivatives have been synthesized, undergone preclinical /
clinical trials (Duncan et al., 2001; Li et al., 1999), some are even marketed. For instance, the PDCs of the N-(-hydroxypropyl) methacrylamide (HPMA) copolymer carrier family, e.g. HPMA –doxorubicin (PK1), HPMA –doxorubicin – galactosamine (PK2), HPMA –paclitaxel, HPMA - platinate and HPMA –camptothecin developed by Duncan et al. (1989 & 1992) have all entered phase I or II clinical trials (Cassidy, 2000; Gianasi et al., 1999; Meerum Terwogt et al., 2001). Meanwhile, the polymer drug conjugate poly-(L)-glutamic acid paclitaxel (PG-TXL) developed by Li C et al. has gone through phase II clinical trial and is marketed as XYOTAX™ (Li et al., 1998, 1999a & b, 2002). Additionally, anticancer PDC Styrene-copmaleic anhydride – neocarzinostatin (SMANCS) has also entered Phase I/II clinical trial (Maeda, 1991).

More examples of PDCs that have been developed and / or patented can be found in Table 2.11.

Table 2.11 Examples of the PDCs that have been developed since 1978.

<table>
<thead>
<tr>
<th>Drug candidates</th>
<th>Aqueous solubility of the drug candidates</th>
<th>Polymer used in drug conjugation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>Insoluble</td>
<td>1-methacryloylamino-2-hydroxypropane</td>
<td>Mongelli et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polyethylene glycols (PEG, linear, branched and dendrimers)</td>
<td>Desai et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly-L-glutamic acid</td>
<td>Li et al., 2002</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>Slightly soluble (0.0392 mg/ml)</td>
<td>Dextran</td>
<td>Brestein et al.1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly-L-lysine</td>
<td>Zunino et al., 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-(-hydroxypropyl) methacrylamide (HPMA)</td>
<td>Duncan et al., 1988</td>
</tr>
<tr>
<td>Drug candidates</td>
<td>Aqueous solubility of the drug candidates</td>
<td>Polymer used in drug conjugation</td>
<td>References</td>
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</tr>
<tr>
<td>Epirubicin</td>
<td>Slightly soluble (0.093 mg/ml)</td>
<td>N-alkyl methacrylamide-based copolymer</td>
<td>Adami et al., 2001</td>
</tr>
<tr>
<td>Fluorouracil</td>
<td>Slightly soluble (&lt; 1 mg/ml)</td>
<td>Poly (α-malic acid)</td>
<td>Ouchi et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclodextrins</td>
<td>Kosak &amp; Keneth, 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dextran</td>
<td>Duncan et al., 2002</td>
</tr>
<tr>
<td>Melphalan</td>
<td>Slightly soluble (&lt; 1 mg/ml)</td>
<td>N-(-hydroxypropyl) methacrylamide (HPMA)</td>
<td>Duncan et al.1991</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Poor solubility (2.5mg/ml)</td>
<td>Dextran</td>
<td>Serino et al., 1988</td>
</tr>
<tr>
<td>Platinum compound</td>
<td>Poor solubility</td>
<td>Diamido-diamine polymer</td>
<td>Duncan et al., 1999a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-(-hydroxypropyl) methacrylamide (HPMA)</td>
<td>Duncan et al., 1999b</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Poor solubility (2.6mg/ml)</td>
<td>Poly-L-lysine</td>
<td>Chu &amp; Howell, 1981</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Soluble (10 mg/ml)</td>
<td>Poly-L-glutamic acid</td>
<td>Hoes et al, 1985.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dextran</td>
<td>Dillman et al.1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Divinylether-maleic anhydride</td>
<td>Pratesi et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(aspartic acid)-polyethylene glycol copolymers</td>
<td>Yokoyama et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(hydroxyethyl-L-glutamine)</td>
<td>Hrelina et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-(-hydroxypropyl) methacrylamide (HPMA)</td>
<td>Duncan et al., 1992</td>
</tr>
</tbody>
</table>
### Drug candidates

<table>
<thead>
<tr>
<th>Drug candidates</th>
<th>Aqueous solubility of the drug candidates</th>
<th>Polymer used in drug conjugation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>Soluble (10-50 mg/ml)</td>
<td>Dextran</td>
<td>Hirano et al., 1980</td>
</tr>
<tr>
<td>Arabinosylcytosine</td>
<td>Soluble</td>
<td>Polygalacturonic acid and carboxyl-methylated yeast β-D-glucan</td>
<td>Kery et al., 1990</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>Soluble</td>
<td>N-(-hydroxypropyl) methacrylamide (HPMA)</td>
<td>Duncan et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly-L-glutamic acid</td>
<td>Singer et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyethylene glycols (PEG)</td>
<td>Rowinsky et al., 2003</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Soluble</td>
<td>Dextran</td>
<td>Matsumoto et al., 1986</td>
</tr>
</tbody>
</table>

Investing further effort in the research and development of the polymer - drug conjugate technology would be of great importance in future treatment of cancer and would definitely yield promising results in the continuous generation of advanced antitumour drug classes.

#### 2.12 Study approach for the current project

At present, substantial work on anticancer polymer - drug conjugate research and development have emphasized on the conjugation of the non water soluble drugs or poorly water soluble drugs to water soluble polymers, with the main intention of increasing the solubility of these drugs in the blood and thus increasing the availability of these drugs to the tumour (Table 2.11). Numerous literatures have been published to document the characteristics and anticancer efficacy of these conjugates. Relatively, the use of water soluble anticancer drugs in synthesizing polymer drug conjugates is less
explored (Table 2.11). Although water soluble drugs do not have the issue of reduced drug anticancer efficacy due to poor water solubility, conjugation of these drugs to the polymeric carriers is also important and necessary. Water soluble drugs often face greater threats in terms of blood degradation by means of hydrolysis or enzymatic action and rapid clearance of the drug molecules from the systemic circulation via renal route due to their good aqueous solubility and smaller molecular weight, compared to the non water soluble drugs or poorly water soluble drugs. At the same time, random dissemination of these drugs into the body tissue compartments may be enhanced due to their good water solubility. All the aforementioned often account for the reduction of these drugs’ availability to the tumour, decrease in the drugs’ anticancer efficacy and the occurrence of the non specific drug toxicity to the normal body tissues during chemotherapy. By conjugating the water soluble anticancer drugs to a biocompatible water soluble polymer, the drugs’ blood degradation and rapid renal clearance may be prevented or delayed (Maeda & Matsumura, 1989; Reynolds, 1995; Seymour et al., 1987). The occurrence of the random drug diffusion events in the body may also be restricted and the concentration of drugs to the tumour site can be increased via EPR effect (as discussed in Subchapter 2.10). Furthermore, development of PDCs using water soluble drugs may face less problems compared to the use of the non water soluble drugs or poorly water soluble drugs in terms of the alteration of the overall water solubility of the synthesized conjugates (the overall solubility of the PDCs may be affected to a greater extent when non water soluble drugs or poorly water soluble drugs are conjugated to the water soluble polymer).

In the current study, we seek to gain further understanding over the outcome of applying the polymer drug conjugation strategy to the drugs of the antimetabolite nucleoside analogue class, an anticancer drug class that has good water solubility, using
one of its most potent members, that is, gemcitabine as a model. Specifically, the alterations over the water solubility, the aqueous and blood stability, the anticancer efficacy and the non specific tissue toxicity of the model drug after conjugating to a polymer through the simple conventional conjugation methods (for example, esterification, amide bond formation) are proposed to be investigated, looking at the fact that most of the functional groups (for example, -OH and -NH$_2$) on the drug molecules of the anticancer nucleoside class that are responsible for the said characteristics may likely be altered during the conjugation reaction. Also, the possible beneficial changes over the in vivo distribution and the tumour targeting properties of the drug upon its conjugation to the polymer were proposed to be investigated, since the small molecular sized antimetabolite nucleoside analogues have the tendency to assume random body distribution once administered in vivo (which contributes to their poor tumour targeting properties). To achieve the above objectives, it was proposed that a novel polymer drug conjugate be synthesized by conjugating gemcitabine to a water soluble linear biopolymer that has been well characterized and frequently employed in PDC synthesis, that is, the poly-L-glutamic acid. Subsequently, the chemical properties and the aqueous and plasma degradation profiles of the synthesized PDC were studied through standard techniques and the in vitro and in vivo anticancer properties of the conjugate were evaluated respectively using a panel of human and mouse breast cancer cell lines and a mouse breast tumour model. The results obtained were then compared to that of the parent drug, i.e. gemcitabine, so as to obtain clues to the abovementioned query and to determine the suitability and potential in employing the polymer-drug conjugate strategy to enhance the antitumour efficacy of the rest of the members found in the water soluble antimetabolite nucleoside class, e.g. troxacitabine, floxuridine, cytarabine, capecitabine, cladribine, clofarabine, fludarabine and pentostatin. A brief account on the poly-L-glutamic acid and gemcitabine are given in the following subchapters.
2.13 Poly-L-glutamic acid

Poly-L-glutamic acid (PG) is a biocompatible, water soluble and biodegradable polymer that has many functional groups (-COOH) for drug attachment. The polymer is composed of a series of L-glutamic acid monomers linked together through an amide bond. It can be found existing in the forms of linear or branched polymers, or dendrimers. Poly-L-glutamic acid can be synthesized by reacting poly (γ-benzyl-L-glutamate with hydrogen bromide (Idelson & Blout, 1958) or through hydrolysis of poly (L-methyl glutamate) with alkaline (Hanby et al., 1950). In a salt free solution, the polymer assumes random coil conformation at pH 7.0 and α-helix rod like conformation in pH < 5.0 (Dolnik et al., 1993; Tsutsumi et al., 1978). The polymer was found to have increase susceptibility to lysosomal degradation, typically to the enzyme cathepsin B (Chiu et al., 1997; Kishore et al., 1990; McCormick-Thomson et al., 1989a).

When injected into the blood circulation for drug carrier purposes, the solubilized PG macromolecules are eliminated primarily through the renal route with limited deposition into the cells of the reticulo-endothelial system. However, the biodistribution and elimination of the PG in the body are still very much dependent on the drug attached to it, the degree of modification of the carboxyl groups of the PG (due to drug attachment) and the rate of PG degradation in blood (in cases where the size of the PG is larger than the renal filtration threshold) (Bayley et al., 1993). In general, PG was found to be non toxic, biocompatible and non immunogenic in humans (Kenny et al., 1959; McCormick-Thomson et al., 1989b; Sumi et al., 1992). It has also been shown to be well tolerated by the human receiver at a single dose of up to 800 mg/kg or multiple doses accumulating at 1.8g/kg (Li et al., 1998 & 1999b).
The first report of PG being employed in PDC development was in 1974 (Batz et al., 1974). To date, PG is still a favourite choice among the research community for a polymeric candidate in the development of PDCs. The anticancer PDCs synthesized using PG as a drug carrier include PG-doxorubicin (Hoes et al., 1985 & 1993; Zunino et al., 1989), PG-daunorubicin (Hurwitz et al., 1980), PG-Ara-C (Kato et al., 1984), PG-Uracil (Mochizuki et al., 1985), PG-cyclophosphamide (Batz et al., 1974), PG-melphalan (Morimoto et al., 1984), PG-paclitaxel (Li et al., 1998, 1999a & b), PG-camptothecin (Singer et al., 2000 & 2001; Zou et al., 2001) and PG-mitomycin C (Roos et al., 1984).

In this study, the PG was selected to be the polymeric carrier for the antimetabolite nucleotide analogue drug in the making of the new polymer-drug conjugate. Compared to other biocompatible polymers, poly-L-glutamic acid has the advantage of being biodegradable (which eliminates the possibility of unwanted polymer accumulation in the body after the drug is released) and has multiple attachment sites (which means greater opportunity for a drug to attach to it) (Li et al., 2002). The linear random coiled conformation of the poly-L-glutamic acid also enables the polymer to be better retained intratumourally than more globular proteins and polymer drug carriers, such as albumins and dendrimers (Gianasi et al., 1999; Henry, 2002; Malik et al., 1999). Besides, the PG’s has abundant drug attachment points (-COOH groups) at its side chains whereby the drug compounds can be conveniently attached to it through simple chemical reactions (Li et al., 2002), which make it a definite good option to be employed for PDC development. Furthermore, the fact that PG has been well characterized and frequently employed in PDC synthesis (as discussed previously) also makes it a good reference polymer candidate to be used in
the evaluation of the suitability and potential for employing the polymer-drug conjugate strategy to enhance the antitumour efficacy of a certain drug class.

2.14 Gemcitabine

Gemcitabine, also known as 2′-deoxy-2′,2′-difluorocytidine monohydrochloride (dFdC) is a cytidine analogue that exhibits antitumour activity. It has a molecular weight of 299.66 and appears as white to off-white crystals in solid state (Merck, 2001). Gemcitabine is found soluble in water, slightly soluble in methanol, and insoluble in ethanol and polar organic solvents (Merck, 2001).

In general, gemcitabine kills cancer cells by disrupting the cell DNA synthesis and inducing of DNA damage (DNA strand breaks) (FDA, 1998). The cytotoxic effect of gemcitabine to the cancer cell is mainly attributed to the combined actions of its active metabolites i.e. the dFdC-diphosphate (dFdCDP) and dFdC-triphosphate (dFdCTP). Upon cellular uptake, gemcitabine is rapidly converted into dFdCDP by cellular nucleoside kinases. Following the conversion, the dFdCDP will exert competitive inhibition to the routine synthesis of cytidine triphosphate (CTP) by the ribonucleotide reductase, causing reduction on CTP synthesis and CTP supply in cell. At the same time, the dFdCDP itself is converted into dFdCTP under the catalytic action of the enzyme it occupies. Upon its synthesis, the dFdCTP enters the nucleus and competes with the dCTP for incorporation into the DNA strand during DNA replication and repair. In the presence of reduced dCTP supply (due to the competitive inhibition of dFdCDP on the ribonucleotide reductase), the incorporation of dFdCTP into DNA (in place of the dCTP) is greatly enhanced. Incorporation of dFdCTP into the cellular DNA often brings fatal consequences as only one additional nucleotide is allowed to be added.
to the growing DNA strands following the integration of the dFdCTP into the extending DNA strand (masked chain termination). The DNA synthesis halt induced by the dFdCTP is irreversible and non-repairable by the available DNA repair mechanisms. As a result, DNA strand breaks are induced at each lesion caused by the dFdC. Eventually, the cancer cells will enter into apoptosis or mitotic death due to severe DNA damage.

In the body, gemcitabine is also metabolized intracellularly and extracellularly by cytidine deaminase into the inactive metabolite diflourodeoxyuridine (dFdU). (Abbruzzese et al., 1991; Stomiolo et al., 1997; USP DI, 1999). When administered to humans, gemcitabine has a terminal half life of 0.1 - 1.6 hours (for short intravenous infusion, i.e. < 70 minutes) and 4.1 - 10.6 hours (for long intravenous infusion, i.e. 70 - 285 minutes) (Stomiolo et al., 1997; USP DI, 1999). Presently, gemcitabine is used for treatment of pancreatic / lung cancer breast cancer and other metastatic cancers (ACM, 2002; FDA, 1998; NICE, 2007). The adverse effects of gemcitabine treatment include myelosuppression, mild proteinuria, hematuria, nausea, vomiting, fatigue, diarrhoea, fever and flu-like symptoms (ACM, 2002; FDA, 1998; NICE, 2007).

Here, gemcitabine was chosen as the representative drug candidate for the evaluation of the suitability and potentiality of employing the polymer-drug conjugate strategy to enhance the antitumour efficacy of the antimetabolite nucleotide analogues. Like the rest of the antimetabolite nucleoside analogues, gemcitabine tend to disseminate randomly in the body (which leads to non specific tissue toxicity and reduced gemcitabine dose to tumour) and is prone to degradation in plasma (by plasma enzymes e.g. cytidine deaminase, Lund et al., 1993). In this case conjugation of gemcitabine with the polymer may potentially reduce its adverse effect (which is important in improving the quality of life of the already weak end stage cancer
patients), protect the gemcitabine from plasma degradation (thus prolonging its plasma elimination half life) and enhance its cytotoxic effects against the tumour metastases (as PDCs may concentrate themselves more efficiently in small tumour mass, Modi et al., 2004). These mentioned benefits may potentially apply for the rest of the antimetabolite nucleoside analogues, should the polymer conjugation strategy work on gemcitabine. Meanwhile from the technical aspect, the availability of the –OH group (at the C5’ position of the gemcitabine molecule) (Figure 2.15) on the gemcitabine’s chemical structure, which may potentially react with the –COOH groups on the PG through esterification reaction and get the drug conjugated onto the polymer, would make the drug an attractive candidate in this project.

2.15 Conjugation of gemcitabine to poly-L-glutamic acid through carbodiimide reaction

In this study, the conjugation of gemcitabine to poly-L-glutamic acid was performed through carbodiimide reaction. Carbodiimide reaction is a chemical reaction that is commonly employed in the protein or peptide modification, that is, in formation of ester bonds between the side chains of amino acid monomers of the peptide and other chemical compounds (Li et al., 2002). The chemical agent employed to facilitate the reaction was the N,N'-dicyclohexylcarbodiimide (DCC). DCC is a dehydration agent often used to activate carboxylic acids to form ester bonds with another chemical that has an –OH group on its structure (Neises & Steglich, 1990). During the carbodiimide reaction, additives such as dimethylaminopyridine, N-hydroxybenzotriazole or N-hydroxysuccinimide, are often added to increase yield and decrease side reactions (Neises & Steglich, 1990). In the current study, the carbodiimide reaction for PDC
synthesis was performed with reference to the method proposed by Li et al. (2002). The proposed reaction scheme is presented in Figure 2.15. The resulting new PDC based gemcitabine derivative was named poly-L-glutamic acid- gemcitabine (PG-G).

**Figure 2.15** Proposed reaction scheme for PG-G synthesis
2.16 Evaluation of the anti breast cancer efficacy of the PDC based gemcitabine derivative Poly-L-glutamic acid – gemcitabine (PG-G)

Upon successful PDC synthesis and chemical characterization, the \textit{in vitro} and \textit{in vivo} anticancer efficacy of the synthesized PDC will be evaluated respectively using a panel of human and mouse breast cancer cell lines and a mouse breast tumour model.

The emergence of breast cancer as the one of the major life threatening malignancies around the world and in Malaysia drew our interest in evaluating the effectiveness of the new PDCs in treating this disease. In the year 2002, breast cancer was found to be the 2\textsuperscript{nd} commonest cancer in the world and the most common cancer detected in women (Parkin et al., 2005). It accounts for one in ten of all newly diagnosed cancer cases and one in four newly diagnosed cancer cases in women worldwide (Parkin et al., 2005). Breast cancer is the main cause of death from cancer in women globally. In Malaysia, breast cancer is the most common cancer found in woman, whereby about one in 19 women may acquire this malignancy during their life span (MO, 2006). The following subchapter gives a brief account on the breast cancers.

2.17 The breast cancer

Breast cancers are cancers formed in breast issue. In general, breast cancer can be divided into two subtypes, that is, the ductal carcinomas (cancer of the epithelial cell lining of the milk gland’s duct) and the lobular carcinomas (cancer of the epithelial linings of the lobes of the milk glands) (NCCN, 2006). Depending on the type of tumour, breast cancer cells may proliferate and form lumps (primary tumour mass) in either the ductal or the lobular regions of the breast. Subsequently, cancer invasion into
the surrounding blood vessels and metastasis occurs. The common metastases sites for breast cancers include the lymph nodes, lung, liver and bone (NCCN, 2006). To date, the aetiology of the breast cancer is yet to be determined. However, studies have revealed that women with a hereditary history of breast cancers, especially those who are carriers of the mutated BRCA I and BRCA II genes may have increased risks in acquiring breast cancer (King et al., 2003). Other risk factors include exposure to radiation, consumption of older types of oral contraceptives (with high oestrogen content), a history of benign breast lumps, obesity, high fat diet, early menarche and late menopause (MO, 2006).

Preliminary breast cancer detection can be performed conveniently by women through self examination of the breast (in search of abnormal lumps). Confirmation of the malignancy status of any detected lump is then done through procedures such as physical examination, mammography, ultra sound scan and tissue biopsy of the breast (MO, 2006). Upon confirmation of malignancy, surgery (lumpectomy or mastectomy) is normally performed. During the surgery, the axilar lymph nodes are checked for presence of metastasis (NCCN, 2006). Usually, radiotherapy and / or chemotherapy (e.g. treatment using combination of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), 5-fluorouracil, doxorubicin, cyclophosphamide (FAC), docetaxel, doxorubicin, and cyclophosphamide (TAC), paclitaxel and gemcitabine, etc.) are / is performed post surgery, no matter for the purpose of clearing up possible cancerous tissue remains at the surgical site, anti-micrometastases or to improve the quality of life of the patient (NCCN, 2006). Occasionally, anti-estrogen treatments (e.g. tamoxifen) may be given to patients with estrogen receptor positive (ER+) tumour (NCCN, 2006). Alternatively, antibody treatment (such as Herceptin, a monoclonal antibody against ERbB2 growth factor receptor) may also be given to the patient (NCCN, 2006).
2.18 The objectives of the current project

As mentioned previously, a poly-L-glutamic acid based polymer anticancer drug conjugate (PDC) that contains gemcitabine as an active antitumour component is proposed to be synthesized and tested against breast cancer. The specific objectives of the study are as follows:

a) To develop a novel poly-L-glutamic acid based PDC containing gemcitabine.

b) To characterize the chemical properties of the synthesized PDC.

c) To determine the \textit{in vitro} and \textit{in vivo} anti-breast tumour efficacy of the synthesized PDC.

d) To study the changes over the \textit{in vivo} distribution of the gemcitabine upon its conjugation to the poly-L-glutamic acid polymer.

e) To evaluate the effect and the usefulness of the application of polymer drug conjugation strategy in improving chemical properties and the antitumour efficacy of the antimetabolite gemcitabine, by comparing the chemical properties and antitumour efficacy of the conjugated gemcitabine to that of the unbound gemcitabine.

f) To extrapolate the potential of applying the polymer-drug conjugation strategy in improving the tumour targeting properties and antitumour efficacy of the drugs of the antimetabolite nucleotide class using gemcitabine as a model.

The details of the methodologies employed in the current study as well as the related results and discussions can be found in the later chapters.