CHAPTER VII

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7.1 INTRODUCTION

The concept of drug targeting, also called the "magic bullet", comes from the experience of the 19th century German chemist, Paul Ehrlich, who selectively stained bacteria for histological examination.\(^1\) The "magic bullet" as an entity comprises two components: one should recognize and bind the target, while the other should provide a therapeutic action in this target.\(^2\) There has been intensive research into the development of biodegradable and biocompatible nanoparticles (diameter <100 nm) as effective drug delivery systems, especially for chemotherapy and gene delivery in cancer treatment.\(^3\) Cancer is caused by the uncontrolled growth and spread of abnormal cells. It is estimated that approximately 11 million people were diagnosed with cancer worldwide in 2003 and an additional 1.3 million new cases were diagnosed in 2004. More than half a million deaths are caused annually by cancer, \(i.e.,\) one in every four deaths. The overall costs for cancer treatment in the United States in 2004 were approximately $190 billion.\(^4\) Effective treatments include surgery, radiotherapy, chemotherapy, hormone therapy and immunotherapy. Each of these treatment modalities has advantages and disadvantages and a combination is usually needed to produce the most effective results. A major obstacle for successful chemotherapy is the resistance of cancer cells to effective anticancer drugs and the destructive action of these drugs on normal cells, tissues and organs\(^5\). For example, the commonly used anticancer drugs, including paclitaxel, doxorubicin, fluorouracil (5-FU), cisplatin and Tamoxifen, are toxic to both tumor and normal cells\(^6\), so the efficacy of chemotherapy is often limited by severe side-effects. In summary, the problems frequently occurring with many drugs include: i) poor solubility; ii)
insufficient in vitro stability (shelf-life); iii) low bioavailability; iv) short in vivo stability (half-life); v) strong side-effects; vi) regulatory issue/hurdles; and vii) lack of large scale production. It has been noted that the disadvantage of most chemotherapies is the relatively non-specific and induced side effects in healthy tissue. Usually, a pharmaceutical agent will distribute evenly within the body. However, ideal chemotherapeutics require a high local concentration of the drug at the disease site(s), while the concentration in other non-target organs and tissues should be below a certain minimal level to prevent any negative side-effects.\(^7\)\(^8\) The poor solubility of certain drugs makes an appropriate delivery system necessary, while undesirable side effects require the technology for site-specific delivery (i.e., drug targeting). One of the main challenges in chemotherapy is the dosage, which for the most effective schedule is determined by the toxicity\(^9\)\(^10\) of the anticancer drugs used. Therefore smart delivery systems\(^11\) are required.

Current research is actively aimed towards achieving specific and targeted delivery\(^12\) of anticancer agents through new avenues of directing drugs to tumors, as well as new types of drugs. Nanotechnology has the potential to revolutionize cancer diagnosis and therapy.\(^13\) Several therapeutic nanocarriers have been approved for clinical use. However, to date, there are only a few clinically approved nanocarriers that incorporate molecules to selectively bind and target cancer cells. Different pharmaceutical carriers, including soluble polymers, microcapsules, microparticles cells, cell ghosts, lipoproteins, liposomes and micelles, have been recently used to multiply the number of drug molecules per single targeting.\(^14\)\(^15\)\(^16\) All of them can assist targeting in one way or another.

To address the challenges of targeting tumours with nanotechnology, it is necessary to combine the rational design of nanocarriers with the fundamental understanding of tumour biology. NPs generally are defined as submicronic (<500 nm) colloidal systems, which refer to a broad spectrum of materials including lipid, polymer and inorganic materials. NPs can provide a controlled and targeted way to deliver the encapsulated anticancer drugs, resulting high efficacy and few side-effects.\(^17\)\(^18\)
Nanocarriers can offer many advantages over free drugs. They:

• protect the drug from premature degradation;
• prevent drugs from prematurely interacting with the biological environment;
• enhance absorption of the drugs into a selected tissue (for example, solid tumour);
• control the pharmacokinetic and drug tissue distribution profile;
• improve intracellular penetration\textsuperscript{19,20}.

For rapid and effective clinical translation, the nano-carrier should:

• be made from a material that is biocompatible, well characterized, and easily functionalized;
• exhibit high differential uptake efficiency in the target cells over normal cells (or tissue);
• be either soluble or colloidal under aqueous conditions for increased effectiveness;
• have an extended circulating half-life, a low rate of aggregation, and a long shelf life.

Incorporation of the drug into a particular carrier can protect it against degradation \textit{in vitro} and \textit{in vivo}. Once the drug carrier has concentrated at the target, the drug can be released either \textit{via} enzymatic activity or changes in physiological conditions, such as pH or temperature, and be taken up by the tumor cells. One of the unique features of tumor micro-vessels is their leakiness as a result of the discontinuity of the endothelium, which facilitates the extravasation of the carrier ingredient and, subsequently, the release of the medication\textsuperscript{21,22}.

Particles, such as micelles and liposomes, ranging from 10 to 500 nm in size, can extravasate and accumulate inside the interstitial space due to the increased vascular permeability in this area. If these particles are loaded with a certain drug, they can bring this drug into the "leaky" zone where it can be released as a result of normal carrier degradation\textsuperscript{23,24}. Since the "cut-off" size of the permeabilized vasculature can vary from case to case, the size of a drug-carrying particle may be used to control the...
efficacy of such spontaneous or "passive" drug delivery by utilizing the EPR effect.\textsuperscript{25,26} Nanocarriers are nanosized materials (diameter 1–100 nm) that can carry multiple drugs and/or imaging agents. Owing to their high surface-area-to-volume ratio, it is possible to achieve high ligand density on the surface for targeting purposes. Nanocarriers can also be used to increase local drug concentration by carrying the drug within and control-releasing it when bound to the targets. Targeting agents can be broadly classified as proteins (mainly antibodies and their fragments), nucleic acids (aptamers), or other receptor ligands (peptides, vitamins, and carbohydrates).\textsuperscript{27,28}

Nanocarriers have been explored for a variety of applications such as drug delivery, imaging, photothermal ablation of tumours, radiation sensitizers, detection of apoptosis, and sentinel lymphnode mapping.

However, inorganic particles may not provide advantages over other types of nanoparticles for systemic targeting of individual cancer cells because they are not biodegradable or small enough to be cleared easily, resulting in potential accumulation in the body, which may cause long-term toxicity. Inorganic NPs generally possess versatile properties suitable for cellular delivery, including wide availability, rich functionality, good biocompatibility, potential capability for targeted delivery (\textit{e.g.}, selectively destroying cancer cells but sparing normal tissues) and controlled release of the carried drugs. Much effort has been put into the surface modification\textsuperscript{29} of biocompatible inorganic particles. Modified NPs as carriers of drugs and biomolecules have several particular advantages in driving loaded drugs to the target site. On the other hand, physical targeting is another promising method to drive drug molecules to recognize targeted cells. It is either based on the abnormal environment (\textit{e.g.}, pH, temperature)\textsuperscript{30} in a target zone, \textit{e.g.}, tumor or inflammation (pH- and temperature-sensitive drug carriers); or magnetically targeting certain cells by driving drug-loaded biocompatible magnetic nanoparticles (MNPs).\textsuperscript{31} External magnetic forces can be applied to concentrate the organic/inorganic nanohybrids to a targeted region and thus increase their chance of interacting with the target cells. This intelligent carrier can decrease the concentration of the drug at non-target sites, thus
reducing side-effects and increase the concentration of the drug at the target site, thus promoting its efficacy.

The magnetic particles injected into an animal were retained at the target site and apparently delayed the reticuloendothelial clearance and neodisposition of the drug-containing carrier. External high-gradient magnetic fields are used to concentrate the complex at a specific target site within the body.\textsuperscript{32}

However, most inorganic MNPs have to be subjected to chemical and/or biological modification to meet the stringent requirements for cellular delivery, such as good biocompatibility, strong affinity between the carriers and biomolecules, high charge density of the nanohybrids, site specificity, etc. Thus, the resulting organic/inorganic nanohybrids can be directly delivered into cells.

\textbf{7.2 PRODRUG ACTIVATION BY ENZYME, A PROMISING STRATEGY FOR CHEMOTHERAPY}

Chemotherapy is an important treatment for cancer patients. However, its success is limited by several drawbacks, including insufficient drug concentrations in tumors, systemic toxicity, lack of selectivity for tumor cells over normal cells, and the appearance of drug-resistant tumor cells. A number of strategies have been used to overcome these problems, including alternative formulations (e.g., liposomes), resistance modulation (e.g., PSC833), antidotes/toxicity modifiers (e.g., ICRF-187), and gene therapy. One promising area for improving tumor selectivity is enzyme prodrug therapy.\textsuperscript{33} Prodrugs are compounds that need to be transformed before exhibiting their pharmacological action. The term prodrug was introduced in the late 1950s by Albert (1958). Enzyme-activating prodrug therapy is a two-step approach. In the first step, a drug-activating enzyme is targeted and expressed in tumors. In the second step, a nontoxic prodrug, a substrate of the exogenous enzyme that is now expressed in tumors, is administered systemically. The net gain is that a systemically administered prodrug can be converted to high local concentration of an active anticancer drug in tumors.\textsuperscript{34} Prodrugs are nontoxic in their native state but are
converted to toxic products by appropriate enzymes. Their effectiveness in treating cancer depends either on enzymes that are highly expressed in malignant cells or on foreign activating enzymes that are targeted to tumors. Another problem is that several human enzymes are relatively inefficient in the activation of the respective prodrugs. In an attempt to overcome this problem, ADEPT, GDEPT, and VDEPT strategies\textsuperscript{35,36,37} enabling the use of exogenous enzymes (e.g., from bacteria and fungi), have been explored. ADEPT, GDEPT, and VDEPT strategies are also used to increase tumor or organ selectivity as shown for treatment of brain tumors with cyclophosphamide.

The rationale for the development of pro-drugs relies upon delivery of higher concentrations of a drug to target cells compared to administration of the drug itself. In the last decades, numerous prodrugs that are enzymatically activated into anticancer agents have been developed. To be clinically successful, both enzymes and prodrugs should meet certain requirements for this strategy. The enzymes should be either of non-human origin or human protein that is absent or expressed only at low concentrations in normal tissues.\textsuperscript{38} The protein must achieve sufficient expression in the tumors and have high catalytic activity. The prodrug should be a good substrate for the expressed enzyme in tumors but not be activated by endogenous enzyme in non-tumor tissues. It must be able to cross the tumor cell membrane for intracellular activation, and the cytotoxicity differential between the prodrug and its corresponding active drug should be as high as possible. It is preferred that the activated drug be highly diffusible or be actively taken up by adjacent nonexpressing cancer cells for a “bystander” killing effect, the ability to kill any neighboring nonexpressing cells.\textsuperscript{39,40} In addition, the half-life of active drug should be long enough to induce a bystander effect but short enough to avoid the drug leaking out into the systemic circulation. Thus, numerous prodrugs of therapeutic agents have been developed to improve their original pharmaceutical, biopharmaceutical, and pharmacokinetic properties.

The combination of indole-3-acetic acid (IAA) and horseradish peroxidase (HRP)\textsuperscript{41} has recently been proposed as a novel cancer therapy. IAA/HRP treatment induces apoptosis in G361 human melanoma cells, whereas IAA or HRP alone have no effect.
Chapter VII

It is known that IAA produces free radicals when oxidized by HRP. Because oxidative stress could induce apoptosis. The HRP/IAA combination shows efficacy in an in vitro tumor model, which is promising for its application in vivo. In particular, the 5Br-IAA prodrug may be important in targeting hypoxia. However, further experiments are required to confirm whether the measured cell kill and potential cell kill are observable in animal growth delay models.

Indole-3-acetic acid (IAA) and horseradish peroxidase (HRP) have emerged as a new strategy for cancer treatment.42

It has been suggested that indole-3-acetic acid (IAA), the plant growth hormone, and horseradish peroxidase (HRP) could be used as a novel cancer therapy. Interestingly, IAA alone was not cytotoxic, but IAA became cytotoxic after reaction with HRP. It has been reported that IAA activated by HRP produces an abundance of free radicals, including reactive oxygen species. We also proposed that hydrogen peroxide plays a major role in IAA/HRP-induced cell death. It has been reported that IAA selectively leads to cell death of human T24 bladder carcinoma cells transfected with an HRP-encoding gene. Activation of the apoptotic signaling pathways is one of the potential mechanisms for the treatment of cancer. It is known that apoptotic nuclear DNA fragmentation occurs during the apoptotic process.43

Several studies have focused on the delivery efficiency and the efficacy of the enzyme pro-drug therapy system by conjugating enzymes on the surface of different nanomaterials. The integration of nanotechnology and enzyme immobilization exhibits several advantages, including high loading capacity, large surface-to-volume ratio, and easy penetration of the plasma membrane. These increase the efficacy of therapy and reduce side effects of therapeutic agents.

Free enzymes show low enzymatic recovery and immunogenicity upon exposure, hence limiting its applications in cancer therapy. One of the possible solutions for the above problem is to shield the epitopes of exogenous enzymes by encapsulating them in porous organic or inorganic matrices. Enzyme immobilization by encapsulation
Chapter VII

plays important roles in enhancing protein stability, facilitating the separation of enzyme and reaction products, allowing multiple or repetitive usage of enzymes, and in establishing a multi-enzyme system for various industrial applications. Enzyme encapsulation in a porous organic or inorganic matrix has been utilized broadly to immobilize and stabilize fragile enzymes.

DIAGRAM SHOWING ACTIVITY OF ENCAPSULATED ENZYME HRP FOR PRODRUG ACTIVATION
In this work, an exogenous horseradish peroxidase (HRP) entrapped in the iron-oxide nanoparticles and manganese phosphate nanoparticles synthesized by microemulsion mediated method were used as \textit{in vitro} enzyme pro-drug therapy system. MCF-7 cell line was exposed to this system. Cytotoxicity due to the interactions with nanoparticles were analyzed using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay method.

7.3 MAINTENANCE OF CELL CULTURE

MCF-7 Cells, Human breast adenocarcinoma cell line, was maintained in complete DMEM medium supplemented with 10% FCS, HEPES (25mM), NaHCO$_3$ (3.7g/liter), penicillin (100units/ml) and streptomycin (100mg/ml). Cells were then grown in the humidified atmosphere of 5% CO$_2$ and 95% air in incubator at 37°C. Cells were kept in the logarithmic growth phase by routine passage every 2-3 days.

Exponentially growing cells were seeded in 96 well plates with a density of 10,000 cells/well and incubated at 37°C for adherence to the bottom of plate. After 18 hrs the media was replaced with 200 µl fresh complete DMEM media. The suspensions of nanoparticles were prepared in distilled water (dH$_2$O) using bath-sonicator. MCF-7 cells were incubated with 50 µM/L of IAA in the presence of 100 µg/ml of both void and enzyme encapsulated manganese phosphate and iron-oxide nanoparticles at 37 °C for 24 hrs and 48 hrs. Triplicates of each sample were set up for the MTT assay and seeded and exposed in an identical manner in case of both manganese phosphate and iron-oxide nano-particles. The experimental conditions and parameters were kept identical with both the nano-particles.
7.3.1 Survival assay:

After exposure to the drug for respective incubation time media was replaced with 180 µl fresh complete DMEM media and 20 µL of MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for additional 5 hrs. at 37 °C. After 5 hrs. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 200 µL of DMSO was added to each well and finally absorbance at 570 and 690 (as background) nm was measured using a microplate reader (Bio Tek Power Wave XS). Triplicates of each sample were analyzed.

Anticancer activity was expressed with respect to the number of viable cells, i.e. anticancer activity is indirectly proportional to the number of viable cells and this in turn is directly proportional to optical density at 570 nm.

**Calculation for Optical density:**

Using the control OD values, the percent of cell viability at each condition of the test agent was calculated as the ratio of mean absorbance of triplicate readings with respect to mean absorbance of control wells:

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\% \text{ Cell viability} = \left( \frac{I_{\text{sample}}}{I_{\text{control}}} \right) \times 100.
\]
In this work the application of magnetic nanoparticles encapsulating enzyme HRP in the enzyme-prodrug therapy (EPT) was explored. We developed a novel EPT system consisting of HRP and the nontoxic plant hormone IAA. For EPT system the enzyme should have high catalytic activity under physiological conditions and fast and efficient prodrug activation even at low concentrations of the substrate (high $K_{cat}$ and low $K_m$), without dependence on further catalysis by other cellular enzymes. In the previous chapters we have shown that HRP in encapsulated conditions has considerable catalytic activity. IAA is well tolerated in humans\textsuperscript{44}, and nonspecific activation in normal tissue is unlikely to take place because mammalian peroxidases.
failed to convert it into a cytotoxin at therapeutically significant prodrug doses. Using this system, we demonstrated fast and efficient \textit{in vitro} prodrug activation. The potential of the HRP/IAA combination to target the tumor vasculature was evaluated using MCF-7 Cells (Human breast adenocarcinoma cell line), as a model. Susceptibility of HRP-expressing MCF-7 cells to prodrug treatment was assessed by exposing the cells to HRP\textsuperscript{+} NP/ IAA and HRP\textsuperscript{-} NP /IAA combinations for 24 hrs. and 48 hrs. MTT assay showing that HRP\textsuperscript{-} PEG-SPION/IAA and MnPi/IAA were not toxic to MCF-7 cells for 24 hrs. On the other hand, significant cell killing was induced in HRP\textsuperscript{+} cells for 24 hrs. exposure. After prolonged incubation upto 48 hrs. with the HRP\textsuperscript{-} and HRP\textsuperscript{+} nanoparticles, the viability of MCF-7 cells reduced from 100\% to 80\% in case of HRP\textsuperscript{-} PEG-SPION/IAA and HRP\textsuperscript{-} MnPi/IAA combinations. This reduction in cell-viability is due to nanoparticles toxicity itself after prolonged exposure to cells. In case of HRP\textsuperscript{+} nanoparticles/IAA, cell viability reduced to 30\% with PEG-SPION/HRP/IAA and 45\% with MnPi/HRP/IAA. From the results, it may concluded that the administered prodrug, which is converted into a potent cytotoxin by the enzyme expressed at the target cells, suggesting the efficacy and selectivity of the HRP/IAA system for enzyme/prodrug-based anticancer approaches.

\textbf{7.4 CONCLUSION}

Current research is actively aimed towards achieving specific and targeted delivery of anticancer agents. In conclusion, the key to cancer therapy is to treat it as early as possible. This requires superior detection and targeting methods combined with nanotechnology. This work explores the recent chemotherapeutic work on drug delivery using nanoparticles as carriers for the targeted treatment of cancer. Compared to direct drug delivery, delivery through a carrier can increase the efficacy of a drug, but decrease the side-effects by utilizing the enhanced permeability and retention (EPR) effect and tumor-specific targeting. In this thesis, we have developed reverse micellar route to generate the enzyme-encapsulated magnetic nanoparticles (EMNPs). The generated enzyme-encapsulated magnetic nanoparticles were uniform, monodisperse and spherical as characterized by transmission electron microscopy (TEM) in previous chapters. In this scheme the enzyme HRP is shielded from the
immune system by encapsulation in nano-particles. Indole-3-acetic acid (IAA) activation by horseradish peroxidase (HRP) has been proposed as a new cancer therapy. Here, we evaluated the potential of enzyme HRP encapsulated magnetic nanoparticles /IAA combination for enzyme/prodrug cancer therapy in human tumor cell line. The feasibility of magnetic nanoparticles (HRP) in the enzyme/prodrug cancer therapy was demonstrated by converting the nontoxic prodrug IAA into the toxic radical products that trigger the cell death of MCF-7 cells. In conclusion, the developed enzyme encapsulated magnetic nanoparticles exhibited several features that make it suitable for future cancer chemotherapy and a variety of biomedical applications.
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Chapter VII


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Chapter VII


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