Development of Multifunctional Poly(glycidyl methacrylate) (PGMA) Nanoparticles for Targeted Cancer Therapy and Imaging

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School of Chemistry and Biochemistry
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Abstract

Nanoparticle based chemotherapy has been predicated to be one of the frontrunners in the field of cancer diagnosis and treatment. Currently, nanoparticle-mediated cancer targeted therapy mainly relies on the abnormal tumor microenvironment for distribution and retention of the nanoparticles. The design and development of multifunctional second-generation nanoparticles seek to expand upon the benefits of these first-generation nanoparticles by combining therapeutic and diagnostic functions within a single formulation and are commonly referred to as “theranostic” agents. These agents can simultaneously deliver imaging probes and therapeutics to specific sites or organs, enabling detection and treatment of disease in a single procedure. Theranostic agents are expected to inform us about localization of the drug and pathological processes longitudinally. This will help make informed decisions about timing, dosage, drug choice, and treatment strategies. However, any additional design feature in drug development will come at the cost of additional regulatory, production, and financial hurdles. The trade-off between these “costs” and clinical benefit will be highly dependent on the choices made when designing drug nanoparticles. Considering these aforementioned criteria, for successful clinical translation, preclinical development of a multifunctional nanoformulation needs to encompass utilization of simple and efficient conjugation methodologies, pertinent yet readily available and cost-effective targeting ligands, and assessment of efficacy in relevant animal models that mimic the clinical situation.

The work presented in this thesis is a systematic progression from development to validation of poly(glycidyl methacrylate) (PGMA) based multifunctional nanoparticles as versatile nanocarriers for receptor-mediated targeted delivery, in models of ovarian and prostate cancers, \textit{in vitro} and \textit{in vivo} respectively. The availability of the epoxide functionality in PGMA enables efficient functionalization through nucleophilic ring opening reactions making PGMA an ideal platform for the conjugation of targeting ligands.
Initial work focuses on the development of folic acid functionalized PGMA nanoparticles to target folate receptor α (FRα) which is overexpressed in ovarian cancer. We introduced a terminal alkyne via an epoxide ring opening reaction of PGMA to enable copper-(I)-catalyzed azide alkyne cycloaddition (CuAAC) with an azide-functionalized folic acid. This possibility of surface chemistry, in an aqueous medium using ambient conditions, without any requirement for tedious purification and by-product removal is advantageous over traditionally used conjugation strategies such as the carbodiimide coupling chemistry. The resulting folic acid functionalized fluorescent nanoparticles and unconjugated nanoparticles were then tested for their targeting capabilities in the FRα overexpressing cell line (SKOV-3, ovarian cancer) and in the FRα deficient cell line (A549, lung adenocarcinoma). Toxicity studies in normal human liver cells confirmed their biocompatibility.

Furthermore, transferrin-functionalized PGMA nanoparticles encapsulating docetaxel and iron oxide nanoparticles were synthesized for chemotherapeutic intervention in prostate cancer. These nanoparticles were tested for cellular uptake and anticancer efficacy in vitro in PC3 cell line and in vivo in a clinically relevant orthotopic model of PC-3 prostate cancer. PC3 cells are characteristic of castration-resistant prostate cancer, which is devoid of most prostate cancer markers and vulnerable to development of docetaxel resistance making it a model of oncological emergency in need for alternative targeted therapies. Tumor growth and retardation in animals were monitored using in vivo bioluminescence imaging and the change in animal weights was taken as a determinant of systemic toxic effects of the formulation. Evaluation of these criteria indicated significant efficacy of the transferrin-tethered nanoformulation at a low drug dosage, at which both the clinically used formulation and non-transferrin tethered nanoparticles were sub-optimal for tumor growth inhibition. Importantly, this study demonstrated that dosage is a pivotal parameter, which needs to be taken into account for the assessment of nanoparticle-mediated targeting. At lower doses, the actively targeted nanoparticles have distinctly higher efficacy in tumor inhibition than their passively targeted counterparts. However, the enhanced permeability and retention (EPR) effect of the tumor tissue becomes the dominant factor influencing the efficacy of both passively and actively targeted nanoparticles when they are administered at higher doses. Post necropsy assessment of nanoparticle distribution in the reticuloendothelial system organs (liver, spleen and kidney) performed using ex vivo magnetic resonance relaxometry suggests a predominant hepatic clearance of the nanoparticles.
Overall, the multifunctional PGMA nanoparticles developed in this work provide inherent feasibility of generalization for both the conjugated ligand and the encapsulated cargo and demonstrate efficient ligand attachment. The attachment of a targeting ligand mediates reduction in drug dosage required for \textit{in vivo} efficacy. This significant effect is not accompanied by body weight loss, an indicator of the detrimental systemic events usually associated with clinical anticancer drug formulations. All these features highlight the promising potential of these multifunctional constructs for further investigation and development that can lead to clinical translation.
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Statement of Candidate Contribution

This thesis contains the results of work carried out by the candidate within the School of Chemistry and Biochemistry and the School of Medicine and Pharmacology at the University of Western Australia during the period of March 2011 to August 2014.

The work presented herein contains no material which the candidate has submitted or accepted for the award of another degree or diploma at any university and, to the best of the author’s knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

The thesis contains work prepared for publication some of which is co-authored. The bibliographic details of the work and author contribution are outlined below.

Journal Articles:


   **Statement of Contribution:** IL and YG prepared PGMA; MN assisted with click chemistry; D. Ho assisted with nanoparticle synthesis. All the work outlined in this manuscript, other than polymer synthesis, has been conducted by RS with support from her direct supervisors, LYL, NMS and KSI with regards to experimental design and writing of the manuscript. This comprises the work presented in chapter 2 of this thesis.

**Statement of Contribution:** IL and YG prepared PGMA; RS was responsible for the multifunctional nanoparticle synthesis and characterization including size and zeta potential measurement, drug loading and drug release studies, transmission electron microscopy, SQuID analysis (with support from RCW) and relaxivity studies on nanoparticles; RS was responsible for all *in vitro* toxicity and flow cytometry analysis; RS and MB performed confocal live-cell imaging. The *in vivo* study (Treatment, BLI data collection and necropsy) was conducted at Proqinase GmbH, Freiburg, Germany using supplied formulations, synthesized by RS. Animal organs - kidney, spleen, liver and primary tumor were shipped back to the University of Western Australia where RS conducted *ex vivo* relaxometry study on these organs (with support from MN and MJH). LYL, NMS and KSI supervised the study. RS and KSI were responsible for analysis of all data and writing of the final manuscript. This comprises the work presented in chapter 3 of this thesis.

**Conference presentations:**

1. Ruhani Singh, Mark Norret, Michael J. House, Samantha South, Michael Bradshaw, Diwei Ho, Robert C. Woodward, Timothy St. Pierre, Nicole M. Smith, Lee Yong Lim, & K. Swaminathan Iyer, ‘Understanding the role of Transferrin in Designing Targeted Nanoparticles using an Orthotopic PC-3 Prostate carcinoma model’, *5th International NanoMed Conference, Coogee Beach, Sydney, Australia (June 2014)*.

2. Ruhani Singh, Mark Norret, Michael J. House, Samantha South, Michael Bradshaw, Diwei Ho, Robert C. Woodward, Timothy St. Pierre, Nicole M. Smith, Lee Yong Lim, & K. Swaminathan Iyer ‘Transferrin anchored and non-anchored Multifunctional Nanoparticle Formulations : Evaluation of Chemotherapeutic efficacy in an Orthotopic PC3 Prostate carcinoma model’, *Australian Society for Medical Research Symposium, WA, Australia (June 2014)*.


5. Ruhani Singh, Nicole M. Smith, Lee Yong Lim and K. Swaminathan Iyer; ‘Multifunctional Nanoparticles for Targeted Cancer Therapy and Dual Magnetic Resonance/Optical Imaging’ Sixth International Conference on Advanced Materials and Nanotechnology, Auckland, New Zealand (February 2013).


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ASPGR</td>
<td>Asialoglycoproteins</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>CdTe</td>
<td>Cadmium telluride</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration resistant prostate cancer</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTx</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Copper catalyzed azide-alkyne cycloaddition reaction</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N’- Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate buffered saline</td>
</tr>
<tr>
<td>Dtxl</td>
<td>Docetaxel</td>
</tr>
<tr>
<td>Dtxl-Np</td>
<td>Docetaxel loaded nanoparticle (without transferrin attached)</td>
</tr>
<tr>
<td>EB</td>
<td>Embelin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FA</td>
<td>Folic Acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>United States food and drug administration</td>
</tr>
<tr>
<td>FR</td>
<td>Folate receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HED</td>
<td>Human equivalent dose</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPMA</td>
<td>N-(2-hydroxypropyl)methacrylamide</td>
</tr>
<tr>
<td>h-Tf</td>
<td>holo-Transferrin protein</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively coupled absorption emission spectroscopy</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer-by-layer</td>
</tr>
<tr>
<td>LDM</td>
<td>Low dose metronomic therapy</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mCRPC</td>
<td>metastatic castration-resistant prostate cancer</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>MDR-1</td>
<td>Multi-drug resistant-1 gene</td>
</tr>
<tr>
<td>MEK</td>
<td>Methyl ethyl ketone</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro ribonucleic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerable dose</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National health and medical research council</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxy succinamide</td>
</tr>
<tr>
<td>NIR</td>
<td>Near-infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NS</td>
<td>N-Succinamide</td>
</tr>
<tr>
<td>O/W</td>
<td>Oil in water emulsion</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival of subject (Clinical)</td>
</tr>
<tr>
<td>P13K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td>PAH</td>
<td>Poly(allylamine) hydrochloride</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amido amine)</td>
</tr>
<tr>
<td>PASA</td>
<td>Poly(L-aspartic acid)</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Poly(cyanoacrylate)</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>PCFT</td>
<td>Proton coupled folate transporter</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(caprolactone)</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity Index</td>
</tr>
<tr>
<td>PDLLA</td>
<td>Poly(D,L-lactic acid)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEHA</td>
<td>Pentaethylenehexamine</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethylene imine)</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glutamic acid)</td>
</tr>
<tr>
<td>PGMA</td>
<td>Poly(glycidyl methacrylate)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLG</td>
<td>Poly(D,L-glycolide)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-\textit{co}-glycolic acid)</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>PPI</td>
<td>Poly(propylene imine)</td>
</tr>
<tr>
<td>PPO</td>
<td>Poly(propylene oxide)</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate specific membrane antigen</td>
</tr>
<tr>
<td>PVP</td>
<td>Poly(vinyl pyrrolidine)</td>
</tr>
<tr>
<td>QDs</td>
<td>Quantum dots</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartate</td>
</tr>
<tr>
<td>RhB</td>
<td>Rhodamine B</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to noise ratio</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation of mean</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interference ribonucleic acid</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carriers</td>
</tr>
<tr>
<td>SMANCS</td>
<td>Poly(styrene-\textit{co}-maleic acid)-NeoCarzinoStatin</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single photon emission computed tomography</td>
</tr>
<tr>
<td>SQuID</td>
<td>Superconducting quantum interference device</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Tf-Dtxl-Np</td>
<td>Transferrin attached, docetaxel loaded nanoparticle</td>
</tr>
<tr>
<td>Tf or Tfαn</td>
<td>Transferrin protein</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TTP</td>
<td>Time to progression</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultra Violet - visible range of the spectrum</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>W/O/W</td>
<td>Water in oil in water emulsion</td>
</tr>
</tbody>
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Chapter 1

Introduction and Literature Review

The successful translation of nanoparticle-based therapeutics into clinical applications has provided the impetus for the development of multifunctional nanoparticles. These multifunctional nanoparticles attempt to further improve clinical outcomes by offering new avenues of consolidated therapy and imaging. However, it is of paramount importance to have a balance between the advantageous multi-functionality and the undermining complexity of a multifunctional nanoparticle system. For this reason, development of multifunctional nanoparticles needs to encompass intelligent design parameters along with facile synthesis procedures. Acknowledging the aforementioned criteria, this PhD research was aimed at the fabrication and characterization of novel multifunctional nanoparticles for the targeted delivery of imaging and chemotherapeutic agents. The primary objective was to develop insights into material design and functionalization strategies, physicochemical characterization, pre-clinical efficacy and potential oncological applications of the nanoparticles. Experiments were designed to meet the following specific aims:

1. Development of poly(glycidyl methacrylate) (PGMA)-based novel conjugation methodologies to facilitate clean, efficient and cost-effective functionalization of PGMA nanoparticles for applications in receptor-mediated cancer targeting.

2. Development and physicochemical characterization of multifunctional nanoparticles for actively targeted chemotherapeutic intervention and validation.

3. Validation of as synthesized, multifunctional nanoparticles in (2), in appropriate in vitro cell models and clinically relevant in vivo mouse model for pre-clinical evaluation of their chemotherapeutic efficacy and a dose dependent comparison with equivalent passively targeted nanoparticles and clinical drug formulation.
To achieve the aforementioned aims, a comprehensive exploration of multidisciplinary literature was required, a review of which is presented in this chapter. The foundation was built on an understanding of development of cancer, various classes of chemotherapeutic drugs, with particular emphasis on the biopharmaceutical issues associated with the taxane class of anticancer agents that are applied in ovarian and prostate cancers. Insights were gained into the drawbacks of conventional anticancer chemotherapy, the rationale underpinning the development of nanoparticle-based therapeutics, together with the potential biodistribution advantages these can offer through the combined mechanisms of passive and active targeting. Further literature review focused on how these insights could be employed to guide the fabrication of polymeric nanoparticles, with the discussion in this chapter centering on the merits and limitations of specific examples. Additional reading provides a review of chemical bioconjugation strategies, an understanding of their advantages and pitfalls guiding the selection of a facile methodology for attaching targeting ligands to nanoparticles.

The possibility of integrating multiple functional components in a single nanoparticle has led to the development of multifunctional nanoparticle systems for simultaneous cancer therapy and imaging. Examples of some of the successful multifunctional nanoparticles are put forward and finally, the importance of nanoparticle toxicity testing for any newly developed nanoparticle formulation is discussed. Overall, this chapter provides a broad background on nanoparticles in cancer therapy and imaging while assessing the considerations to be made for synthesis of novel multifunctional nanoparticles. Each of the subsequent experimental chapters in the thesis will begin with their own specific introduction with a short literature review to provide a rationale for development and validation of PGMA multifunctional nanoparticles as discussed in each of the chapters respectively.
1.1 Cancer: An Introduction

1.1.1 Global cancer statistics

Cancer is estimated to be the leading cause of death worldwide with a cumulative mortality rate of 15% in developed and 12.7% in developing nations. The risk of incidence linked with the disease is 30% for developed and 17% for the developing nations. Such disparities in incidence and mortality patterns in the developed and developing countries reflect regional differences in prevalence and distribution of major risk factors, detection practices, and availability and use of treatment facilities. As shown in figure 1.1, breast cancer in females and lung cancer in men are the most frequently diagnosed, and leading causes of cancer related death. In some of the developed nations, lung cancer is outweighed by prostate cancer. There have been significant advances in the field of cancer diagnosis and treatment over the past four decades, however the statistics suggest that presently available modalities are still far from optimal. Given that the global cancer burden is expected to nearly double by 2030, newer and more advanced diagnostic and therapeutic agents need to be developed. For this reason, an understanding of the development of the disease, its progression and presently used treatment modalities is important.

![Figure 1.1 Total estimated new cancer cases and deaths worldwide for leading cancer sites. Figure drawn with data from Jemal et al. 2011.](image)

1.1.2 Development of disease and treatment modalities

The development of cancer is an intricate multistep process that involves accumulation of scores of alterations in genomic structure and function. A normal mammalian cell has an ordered cell division cycle comprising the G1, S, G2, and M phases. The cell
replicates its genetic material in the S phase, which is then segregated into two identical daughter cells in the M phase. The gap periods between the S and M phases allow for metabolic changes to prepare the cell for division (G1 phase), and the institution of ‘checkpoints’ to confirm for appropriate extracellular signals, cell size and genetic material integrity for mitosis (G2 phase).³,⁴ The cell cycle is a very well regulated process that responds to the specific needs of any tissue or cell. Normally, in an adult tissue a dynamic steady state is maintained via regulation of a homeostatic balance between programmed cell death (apoptosis) and cell proliferation (cell division).⁵

The key aspect of the transformation of normal cells into cancerous cells are the genetic and epigenetic alterations that lead to disruption of the aforementioned equilibrium by interference with signaling pathways that control normal cell proliferation, motility and survival (Figure 1.2).⁵,⁶ Specifically, these alterations occur as either (i) Gain-of-function mutations to form oncogenes, the mutant versions of protooncogenes, products of which have a role in positively regulating the signal transduction pathways to promote cell proliferation, or (ii) Loss-of-function mutations affecting the tumor suppressor genes and DNA repair genes, which code for proteins that provide negative feedback to regulate the cell cycle.²,⁷

These genetic mutations disrupt the normal patterns of gene expression, often leading to expression of abnormal and constitutively active proteins and the epigenetic changes deregulate mechanisms such as transcriptional control leading to the inappropriate silencing or activation of cancer-associated genes.⁸

Genetic mutations could result from a wide range of structural changes in the DNA, including change in chromosomal copy numbers, chromosomal alterations such as translocations, deletions, amplifications and changes in nucleotide sequences as well as point mutations affecting single nucleotides at an important location of a cancer-associated gene.⁹ For example, the mutation and loss of function of the TP53 tumor suppressor gene is one of the most frequent genetic abnormalities, observed in almost every type of cancer, at rates varying from 38-50% in ovarian, esophageal, colorectal, head and neck, larynx, and lung cancers to about 5% in primary leukemia, sarcoma, testicular cancer, malignant melanoma, and cervical cancer.¹⁰ TP53 encodes for protein p53, which is a transcription factor that regulates expression of multiple genes involved in the anti-proliferative responses under conditions of cell stress in particular, DNA damage.⁸,¹⁰ For this reason, a somatic mutation in the gene could result in untimely cell
proliferation in the presence of genotoxic conditions. Another example is the epidermal growth factor receptor (EGFR) family consisting of four genes, EGFR (ErbB1), HER2 (ErbB2), EGFR3 (ErbB3) and EGFR4 (ErbB4), which are responsible for proliferative and anti-apoptotic responses in the cell.\(^8\) However, many brain cancers and epithelial cancers such as squamous oral or esophageal cancers are associated with amplification of EGFR, and HER2 is frequently overexpressed in breast and ovarian cancers.\(^11\) These activating EGFR mutations lead to alteration in structure of a region of tyrosine kinase (TK) domain which is involved in ATP binding. This confers ligand independence and mutational activation of downstream Akt and STAT signaling pathways, which promote cell survival and induction of dependence on EGFR signals.\(^12\)

In a normal cell, epigenetic mechanisms maintain cell differentiation processes via DNA methylation, histone modification and RNA-mediated silencing.\(^13,14\) These mechanisms are essential for stable propagation of gene activity from one generation of cell to another. However, an alteration in these mechanisms leads to inappropriate gene expression resulting in development of cancer.\(^15-17\) For example, nearly 50 genes have been reported to be hypermethylated in prostate cancer.\(^18,19\) Amongst these, at least 12 genes have consistently been found to be affected in a vast majority of cases.\(^20\) For this reason, a combined hypermethylation assay for a group of genes, RASSF1A, RARB2, APC, GSTP1 or GSTP1, APC and MDR1, can very robustly discriminate between benign and tumorigenic changes in the prostate.\(^21,22\)

Clonal populations with these genetic and epigenetic mutations are subjected to Darwinian natural selection leading to attainment of eight functional capabilities\(^7\); (1) self-sufficiency in growth signalling, (2) insensitivity to growth-inhibitory (anti-growth) signals, (3) evasion of programmed cell death (apoptosis), (4) limitless replicative potential, (5) sustained angiogenesis, (6) tissue invasion and metastasis, (7) capability to modify or reprogram cellular metabolism (abnormal pathways) and (8) ability to evade immunological destruction. These functionalities are the ‘hallmarks of cancer’ that dictate malignant growth and lead to the development of a primary tumor in a particular organ or tissue site.\(^7,23,24\)
Figure 1.2 Major signaling pathways and the onset of cancer. The onset of cancer involves alterations to intricate and complex pathways in the cell that control and monitor gene expression, cell proliferation, DNA integrity, and cell death. Some of the genes known to be functionally altered are highlighted in red. Figure from Hanahan and Weinberg 2000.²
Once the tumor is formed, cancer cells can then recruit normal cells, in particular bone marrow-derived cells in the tumor microenvironment, to aid in their dissemination from this primary site, their survival in circulation, and the infiltration, seeding and establishment of new cancer sites in distant tissues and organs, a phenomenon known as metastasis.\textsuperscript{25} Macrophages can increase the metastatic potential of cancer cells by facilitating tissue invasion and intravasation, as well as angiogenesis. Myeloid cell-derived suppressor cells and blood platelets suppress the immune response to tumor antigens in circulation, and have an important role in the activation of microthrombi (the clotting system) to help lodge the cancer cells into new organs and tissues.\textsuperscript{26}

Following metastases, it becomes even more challenging to treat cancer. The small size of metastases makes them inaccessible to surgical removal and undetectable by imaging. Prognosis becomes poorer due to the high multiplicity characteristic and involvement of diverse ectopic organ environments in this diseased state.\textsuperscript{23}

On diagnosis, the general approach for cancer management is tumor eradication using one or more treatment modalities, which may include surgery, chemotherapy, radiation therapy, hormonal therapy and relatively newer and costlier targeted molecular treatments like monoclonal antibody (mAb)-based immunotherapy. The appropriate choice of therapy to be undertaken depends on a number of factors, including the general health status of the patient, and the location and stage of the cancer. In the initial stages, when the tumor has not metastasized or is hormone-dependent, local treatments such as surgery and radiotherapy or hormonal manipulation therapy can be used. However, with the propensity of cancer cells to enter adjacent tissues, systemic treatment using chemotherapeutic agents is often required, either alone or in combination with the other treatments. There is now a wide spectrum of chemotherapeutic drugs available, with more potent drugs getting approved for the management of cancer.

1.1.3 Chemotherapy (CTx): The chemical approach for cancer management

The word \textit{chemotherapy} was coined by the famous German chemist Paul Ehrlich in the early 1900s and was originally meant for the use of chemicals to treat a disease.\textsuperscript{27} It has since evolved to become more narrowly associated with cancer therapy. Drug discovery for cancer treatment dates back to the 20\textsuperscript{th} century and was serendipitous.\textsuperscript{28} During the World War II, an accidental spill of sulfur mustards, which had been used since World
War I for chemical warfare, was found to cause the exposed men to subsequently develop markedly depleted bone marrow and lymph nodes. This led to experiments on mice to examine the potential therapeutic effects of nitrogen mustard. The first successful human study, published in 1946, initiated the synthesis and evaluation of alkylating agents for cancer treatment.

Since then, many more chemotherapeutic drugs have been developed, and are used either as a single agent, in combination with one or more other drugs (combination chemotherapy), or in combination with other treatment modalities, like radiotherapy (chemo-radiotherapy). Chemotherapeutic agents induce cytotoxicity by programming cell death or apoptosis. Depending on the mechanism and the biological molecules they affect, chemotherapeutics can be classified into the categories of:

1. Alkylating agents – e.g. cyclophosphamide, lomustine, carmustine, dacarbazine, mitomycin, cisplatin and derivatives (carboplatin, oxaliplatin);
2. Antimetabolites – e.g. methotrexate, pemetrexed, fluorouracil, gemcitabine, thioguanine and mercaptopurine;
3. Protein kinase inhibitors – e.g. erlotinib, gefitinib, lapatinib, nilotinib, and sunitinib;
4. Topoisomerase inhibitors – e.g. etoposide, doxorubicin, mitoxantrone, teniposide, and aclarubicin;
5. Antibiotics – e.g. daunorubicin, doxorubicin, epirubicin, idarubicin, mitaxantrone, and,
6. Anti-microtubule agents – e.g. vincristine, vinblastine, paclitaxel and docetaxel.

The aim of this thesis was to develop nanoparticle-based formulations appropriate for the treatment of ovarian and prostate cancer. These cancers are particularly considered to be oncological emergencies because of the availability of only sub-optimal chemotherapeutic treatments and their tendency to develop into a refractory disease. Chemotherapy regimens currently approved by the US Food and Drug Administration (FDA) for the treatment of these cancers encompasses the clinical formulations of anti-microtubule agents called taxanes, namely paclitaxel and docetaxel. The mode of action of these drugs and the problems associated with their clinical formulations are reviewed in the next section.
1.1.4 Taxanes: The anti-microtubule chemotherapeutic agents

The history of taxanes dates back to the 1970s when the extract from the bark of the western yew tree, *Taxus brevifolia*, was found to exhibit anti-tumoral activity against murine tumors. Wani *et al.* identified the compound responsible for this activity as paclitaxel as shown in figure 1.3. Despite its high potency, paclitaxel was taken off market for about a decade because of concerns centering around the exploitation of a limited resource (yew trees) to meet demands, and hypersensitivity reactions related to its clinical Taxol® formulation. Later, a semisynthetic analogue called docetaxel (Figure 1.3) was synthesized by esterification of 10-deacetylbaclatin III, a parent molecule which by itself had no inherent cytotoxic activity. 10-deacetylbaclatin III was extracted from the needles of the European yew tree *Taxus baccata*, which is a more renewable source. Eventually, semisynthetic paclitaxel also became available. Since then Taxol® and Taxotere®, the approved formulations of paclitaxel and docetaxel, respectively, have been clinically used against a wide variety of tumors, especially breast, ovarian, prostate, gastric, non-small-cell lung, and head and neck cancers.

![Figure 1.3 Chemical structure of taxanes: Paclitaxel (left) and Docetaxel (right).](image)

The taxanes inhibit cell proliferation by stabilizing cellular microtubules (Figure 1.4A-C). Taxanes bind to microtubules and prevent their disassembly in a guanosine triphosphate-independent manner. This causes disruption of microtubule dynamics leading to the arrest of the cell cycle at the metaphase to anaphase boundary, inhibiting cancer cell proliferation (Figure 1.4D). Another mode of action, known as the low dose metronomic (LDM) therapy has been suggested, whereby the drugs exhibit anti-angiogenic effect if they are administered at low doses on a continuous schedule.
On a molar basis, docetaxel is more cytotoxic than paclitaxel both in vitro and in xenograft models, because of its higher activity as a tubulin assembly promoter and stabilizer. However, there are few published clinical studies comparing the two drugs. A review of the phase II and phase III studies involving the administration of docetaxel or paclitaxel in combination with other anticancer agents suggests that they produce similar outcomes in overall patient survival. The clinical formulations for the two drugs also share similar limitations, which may be divided into (a) hypersensitivity reactions, (b) drug resistance and (c) toxicity.

Paclitaxel and docetaxel are characterized by extremely low water solubilities of 0.3 µg/ml and 4.93 µg/ml, respectively. Consequently, the formulation of taxanes into aqueous solutions of appropriate strength requires solubilizing agents. Taxol® consists of paclitaxel dissolved at 6 mg/ml in 527 mg of polyoxyethylated castor oil (Cremophor EL®) and 49.7% (v/v) dehydrated ethanol. Taxotere® is a solution of docetaxel solubilized at 40 mg/ml using polysorbate 80 (Tween® 80) in a 13% w/w ethanolic aqueous solution. Both the excipients, Cremophor EL® and Tween® 80 are associated with the induction of hypersensitivity reactions in patients. In some patients, the hypersensitivity reactions are mild, manifested as rashes; in others, they can escalate to become severe and life threatening anaphylactic reactions.

Taxanes, more frequently docetaxel are vulnerable to the phenomenon known as multidrug resistance (MDR), which describes the development of resistance in tumor cells to the cytotoxic effects of one or more chemotherapeutic agents after repeated exposure. MDR can develop through a variety of mechanisms. An important mechanism is the expression of transcellular membrane proteins which act as molecular efflux ‘pumps’ in the cancer cells. A well-known example is p-glycoprotein, a member of the ATP-binding cassette transporter proteins that is encoded by the multi-drug resistant-1 (MDR1) gene. These efflux pumps hinder drug accumulation in target cells, and contribute to therapeutic failure.
Figure 1.4 Mechanism of action of taxanes. (A) Shows structure of a microtubule composed of α (light grey spheres) and β tubulin (dark blue spheres), which form a cylindrical structure head to tail in 13 protofilaments. Each microtubule has a (+) end, with β-tubulin facing the solvent, and a (-) end, with α-tubulin facing the solvent. (B) Shows a microtubule cut away to show interior surface. Paclitaxel binds along the interior surface of the microtubule, suppressing its dynamics. (C) Shows that microtubule ends grow and shorten stochastically over time by addition and loss of tubulin subunits from their ends. Figure (C) bottom shows life-history traces of the lengths of four individual microtubules in the absence of drug (left) and in the presence of a microtubule-targeted drug (right). In the presence of anti-microtubule drugs like the taxanes, the dynamics are suppressed. (D) Shows human osteosarcoma cells in different stages of cell cycle without (i-iv) and with (v) addition of antimitotic drugs. Microtubules are shown in red, chromosomes in blue and kinetochores in green, (i-iv) show normal cell cycle: Prometaphase – nuclear envelope breaks down, chromosomes are condensed and dynamic microtubules probe the cytoplasm until they contact a chromosome; Early metaphase – most chromosomes have congressed to the equator to form a metaphase plate; Anaphase – the duplicated chromosomes have separated and are moving towards spindle poles to form two daughter cells; Telophase – separated chromosomes have reached the spindle poles and the cell is dividing to form two daughter cells; (v) Shows that in presence of 10 nM paclitaxel, unlike stage (ii) of a normal cell cycle, some chromosomes remain at the spindle poles and have not congressed to the metaphase plate. This disruption of microtubule dynamics leads to the arrest of the cell cycle at the metaphase to anaphase boundary inhibiting cancer cell proliferation. Figure and caption modified from Jordan and Wilson 2004.46

Apart from the external factors, the taxanes themselves are highly cytotoxic agents. Like most other antineoplastic agents, they do not differentiate between highly proliferative malignant cells and normal fast dividing cells in the body such as blood cells and cells lining the hair follicles, mouth, stomach, intestines etc. resulting in non-specific toxicity.
The most common side effects associated with the taxanes are, mucositis (inflammation and ulceration of the mucous membrane lining the oral and gastrointestinal tracts), neutropenia (deficiency in the number of white blood cells), febrile neutropenia (neutropenia followed by infection and fever), thrombocytopenia (decrease in blood platelet count) and anemia (decrease in red blood cells). These adverse effects limit the upper dose of the taxanes (dose-limiting toxicities), resulting in sub-optimal doses being administered to patients.

Another problem associated with taxane chemotherapy is limited tumor penetration due to entrapment of the drug within the micellar structures of Cremophor EL® and Tween® 80. Entrapment of the drug in the micelles has also been shown to decrease systemic clearance and volume of distribution, leading to a non-linear pharmacokinetic profile and lack of dose-dependent activity of the drug. Other drugs co-administered with the taxane formulations could also be affected by micellar entrapment.

1.1.5 The rationale behind a ‘nanotechnological’ solution to the ‘chemotherapeutic’ problem

Most anticancer drugs, like the taxanes are non-discriminating in their cytotoxic effect, killing both cancer cells and normal cells with rapid turnover rates. This leads to side effects, which may be acute, lasting for hours to days after drug administration, or chronic, which may last from weeks to the entire lifetime of a patient. Common side effects of chemotherapeutic agents include gastrointestinal distress (nausea, vomiting, anorexia, diarrhea, and constipation), myelosuppression and immunosuppression (anemia, thrombocytopenia, neutropenia, and neoplasm), hair loss, water retention, and erythema. Organ damage resulting from cardiotoxicity (heart damage), hepatotoxicity (liver damage), nephrotoxicity (kidney damage), ototoxicity (damage to inner ear) as well as infertility and cognitive impairment have also been reported. Unpredictable yet life threatening hypersensitivity reactions may also result, either due to the drugs themselves (cisplatin, carboplatin, etoposide) or to the excipients used to solubilize the drugs. Furthermore, as most chemotherapeutic drugs are small molecules, they have short plasma half-lives. For this reason, they need to be administered multiple times in a prescribed regimen. Frequent fluctuations of the drug concentration in the blood attunes cancer cells to the drug, leading to the development of MDR, which causes a drug with initial good anti-tumor response to fail on subsequent treatment.
To resolve some of these issues, macromolecules such as monoclonal antibodies, peptides, gene fragments, and proteins have been developed as anticancer agents. While these molecules are considered more specific in their activity compared to low molecular weight drugs, they present their own unique issues, such as susceptibility to premature enzymatic degradation by proteases and DNA/RNAses followed by rapid clearance from circulation. Most of these molecules are regarded as ‘foreign’ by the body’s defense systems and owing to their multi-potency they can stimulate a fatal immune response. Moreover, the targets for most nucleic acid, peptide and protein therapeutic agents are located intracellularly, so the low permeability of the cell membrane to these macromolecules can become an obstacle in the development of their formulations.

All of these issues underscore the rationale for the development of nanoparticle-aided chemotherapy. Nanoparticle-based delivery systems consist of an organic or an inorganic platform that is either conjugated directly to the drug or provides a matrix for drug loading. In either form, the drug availability increases without the use of toxic solvents as vehicles for drug solubilization and administration. The drug is protected from premature degradation and efflux transport as it is not accessible for interaction with degrading enzymes or efflux transporter proteins. For this reason, nanoparticle-based delivery is advantageous for both low molecular weight drugs and macromolecular agents. Nanoparticles can be either passively targeted to a tumor tissue or actively targeted to particular cell type depending on their surface chemistry. Targeting facilitates specificity of action, which in turn improves the pharmacokinetic parameters and tissue distribution of drugs, and by extension their toxicity and immunogenic profiles. Compared to the free drug, drugs encapsulated in nanoparticles have longer circulation half-life, lower renal clearance, and may lead to reduction in the drug dosage required.
1.2 Nanotechnology for cancer chemotherapy

Nanotechnology in its strictest definition by the US National Nanotechnology Initiative refers to structures that have at least one of their dimensions in the 1-100 nanometer (nm) size regime. However, nanotechnology in its current and emerging applications commonly refers to materials of up to several hundreds of nm in size. These materials can be developed either by top-down or a bottom-up engineering of the individual components. It is the physical and chemical properties of nanoscale materials that are advantageous, and these properties are not available both of the bulk material as well as the atoms and molecules of which the material is composed. Furthermore, these novel physicochemical properties are tunable with changes in size and shape of the nanomaterial.

The subcellular compartments and molecular levels of functioning of living cells operate at a nanoscale. This similarity in scale has implication in the utilization of nanoparticles to interact with biological components at the cellular level. Nanotechnology plays a significant role in biology by providing us with the ability to manipulate at the sub-cellular and genetic level and permit these biological interactions specifically at the required intracellular site of action.

Ehrlich was the first to coin the concept of “magic bullet” in 1906 for the targeted delivery of drugs. This concept is particularly pertinent to improve the delivery of conventional small molecule chemotherapeutic agents, which exhibit non-specific activity causing side effects. Out of a certain clinical dose administered to a patient, only a fraction reaches the tumor resulting in drug levels that are sub-optimal for efficacy. However, nanotechnology holds the potential to re-direct the systemic distribution of these drugs towards a particular target site resulting in increased efficiency and circumvention of toxic effects. Two targeting strategies have been applied using nanoparticles – passive targeting to tumor sites and active targeting to cancer cells. These strategies exploit the abnormal tumor microenvironment and the distinct cancer cell molecular expression. The abnormal pathology of tumor vasculature and low pH conditions are particularly relevant targets for nanoparticles.
1.2.1 Nanoparticle-mediated passive targeting of tumor: The Enhanced Permeability and Retention effect (EPR effect)

The mechanism for nanoparticle-mediated passive targeting of the tumor was first reported about 30 years ago when Maeda et al. observed preferential distribution and retention of their novel macromolecular therapeutic, poly(styrene-co-maleic acid)-NeoCarzinoStatin (SMANCS) in the tumor interstitium. The term that they coined, *Enhanced Permeability and Retention* (EPR) effect (Figure 1.5) has since then gone on to become one of the most widely used descriptors for nanoparticle-mediated drug delivery.

Initially, when a tumor is only 1-2 mm$^3$ in size, it can derive oxygen and nutrients *via* the process of diffusion. As the tumor cells multiply, the cluster grows until it reaches a size of about 2-3 mm$^3$. It is then that a state of hypoxia eventuates because of the increased demand for oxygen and nutrition. This state compounded by the longer diffusional distance between the cells and the vasculature initiates the formation of new blood vessels from existing vessels, a process known as *angiogenesis*. The ‘angiogenic switch’, signaling a progression from non-angiogenic to angiogenic phenotype, is essential for continued tumor growth and subsequent metastasis.

The rapid growth of tumor vasculature results in its anatomically unregulated architecture and vessels that are irregularly shaped, dilated, and leaky due to a discontinuous and defective endothelium. This defective endothelium lacks the basal membrane seen in normal vasculature, with resultant fenestrations in the capillaries ranging from 200 to 2000 nm in size, depending on the tumor type and location. Circulating blood components and nanoparticles can readily extravasate in the vicinity of these fenestrations to reach the tumor interstitium. This contributes to the *enhanced permeability* component of the EPR effect.

In normal tissues, there is provision for a continuous drainage of extracellular fluids into lymphatic vessels to allow for the recycling of extravasated solutes back into circulation. The lack of lymphatic vessels in tumor tissue limits the fluid drainage and the return of extravasated solutes back into circulation by convective forces. Diffusion remains operational, but is accessible only to small molecules. Consequently, nanoparticles that have extravasated into the tumor from perivascular spaces accumulate in the tumor interstitium. This contributes to the *enhanced retention* component of the EPR effect.
**Figure 1.5 Active and passive tumor targeting.** Schematic representation of different mechanisms by which nanoparticles can deliver drugs to tumors. Polymeric nanoparticles are shown as blue circular structures. Passive tissue targeting is achieved by extravasation of nanoparticles through increased permeability of the tumor vasculature and ineffective lymphatic drainage (EPR effect). Active cellular targeting (inset) can be achieved by functionalizing the surface of nanoparticles with ligands that promote cell-specific recognition and binding. The nanoparticles can (i) release their contents in close proximity to the target cells; (ii) attach to the membrane of the cell and act as an extracellular sustained-release drug depot; or (iii) internalize into the cell. Figure and caption from Peer *et al.* 2007.

### 1.2.1.1 Abraxane®

Abraxane® is an example of clinically successful therapeutic application of passively targeted nanoparticles. As shown in figure 1.6, it is a nanoparticle based formulation constituting of ~ 130 nm (diameter) sized albumin-bound paclitaxel nanoparticles. Abraxane® was first approved by the FDA in 2005 for the treatment of refractory metastatic breast cancer and has since then been approved for the treatment of non-small-cell lung cancer (2012) and advanced pancreatic cancer (2013).

Abraxane® demonstrated higher penetration into tumor cells, increased efficacy and a maximum tolerable dose (MTD) that was 70% higher than Taxol®. None of the patients in the Phase I clinical trial experienced hypersensitivity reactions, while the
toxicities in a Phase II trial were generally less frequent and less severe for Abraxane® compared with Taxol®. In a large multicentre phase III trial, the overall response rate for Abraxane® was significantly superior to Taxol® (33% vs 19%, \( p = 0.001 \)) and there were significantly lower incidences of toxicity despite the administration of Abraxane® at a 50% higher dose than Taxol®.

![Figure 1.6](image)

**Figure 1.6** Abraxane® (nab™-paclitaxel) is an assembly of individual paclitaxel bound albumin molecules into a ~130 nm sized nanoparticle. Each single molecule of albumin can bind up to 6 or 7 molecules of paclitaxel. Figure from website for Abraxane®.

However, these advantages are offset by its higher cost, and when expense is taken into consideration, the improvement in antitumor efficacy is regarded as only marginal for Abraxane®. The median time to progression (TTP) and median patient survival time are longer with Abraxane® than with Taxol®, but this benefit is only in the time-frame of weeks. This provided the impetus for Karmali *et al.* to modify the passively targeting Abraxane® by introducing two peptides for active targeting. Preliminary results are promising, showing a significantly increased efficacy of the active targeting nanoparticles versus Abraxane® in mice bearing the MDA-MB-465 human breast cancer xenograft.

### 1.2.2 Nanoparticle-mediated active targeting of tumor

Active targeting mechanisms program the nanoparticles to specifically bind to cancer cells after extravasation. The EPR effect by itself may not be adequate for targeting drug delivery to all tumors, for instance some solid tumors have lower permeability and highly heterogeneous vasculature, which can cause unpredictable drug deposition. It may therefore be helpful to attach specific ligands to the nanoparticles to actively target and enhance their internalization in cancer cells *in vivo*. Tumor cells and associated
vasculature can be actively targeted by attachment of ligands to nanoparticles that would recognize and bind to specific targets that are expressed on cell membrane or blood vessel endothelium as shown in figure 1.7. Especially for drugs such as the taxanes that act on intracellular targets for their mode of action, selective internalization of the drug loaded nanoparticles into cancer cell is important to increase their accumulation in diseased tissue while decreasing systemic distribution and side effects.

Figure 1.7 General design of an active targeting nanoparticle-based drug delivery system. Polymer-based engineered nanoparticles offer immense diversity in terms of the nature, properties, structure, composition and ease of functionalization. The possibility of loading (or conjugation) with a drug and functionalization with specific ligands, intended for recognition by receptors overexpressed at the surface of specific cells, such as cancer cells, brain endothelial cells etc. has paved the way to actively targeted therapy. The successful design of an actively targeted nanoparticle is governed by nature of the polymer and the ligand (vitamins, hormones, peptides, proteins, etc.), as well as various strategies to conjugate and display them on surface without altering their biological activities. Figure and caption from Nicolas et al. 2013.106

The choice of active targeting ligand depends on whether the therapy is being targeted to control (a) cancer cell proliferation or (b) angiogenesis.91 It also depends on the target molecule, which is usually a cancer specific marker or a receptor overexpressed on the target cancer cells.106 Targeting ligands can be broadly divided into (1) proteins – which include antibodies, antibody fragments and peptides; (2) nucleic acids – which include aptamers; and (3) others – which include small molecules and carbohydrates.96
A wide range of targeting ligands have been reported in literature, amongst which antibodies have emerged as the most potent and successful agents. Over 17 antibodies have already been approved by the FDA for oncological indications and many more are presently being investigated in clinical trials. Antibodies present with high efficiency because of their high specificity, selectivity and affinity for the tumor antigen. Initial clinical applications utilized antibodies as antagonists of the oncogenic receptor tyrosine kinases, epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGF) and erythroroblastic growth factor receptor HER2/ErbB2. Examples include Cetuximab (anti-EGFR), Panitumumab (anti-EGFR), Trastuzumab (anti-HER2) and Pertuzumab (anti-HER2). The first angiogenic inhibitor, Bevacizumab, was an anti-VEGF mAb. Antibody fragments have also been used and regarded as safer alternatives to whole antibodies because of their lower capacity for non-specific binding and immune activation. However, whole antibodies have been suggested to have higher avidity and stability. Overall, both mAb and antibody fragments have a common drawback of being expensive and requiring expertise for production.

Researchers have attached both mAb and antibody fragments to nanoparticles imparting them with tumor-targeting capabilities. In one such study, Kirpotin et al. provided interesting data that distinguishes between the passive and active targeting effects of nanoparticles in a BT-474 breast cancer xenograft rodent model. They compared the uptake of liposomes loaded with gold nanoparticles and surface-functionalized with poly(ethylene glycol) (PEGylated), with and without an anti-HER2 mAb conjugated onto them. The authors found a 6-fold increase in the intracellular uptake of the liposomes conjugated with the antibody, but there were no differences in the tumor tissue accumulation of both the liposomal formulations. While both types of liposomes were successfully directed to the tumor by passive targeting, the antibody-conjugated liposomes were internalized by the cells whereas the non-targeting liposomes were predominantly located in the extracellular stroma or macrophages.

Proteins have also emerged as a good targeting modalities. Taking the transferrin receptor as a pertinent example, the higher demand of nutrients including iron in cancer cells often leads to an overexpression of the transferrin receptor (TfR). TfR overexpression has also been associated with states of metastasis and drug-resistance in solid tumors, and therapeutic failure in brain gliomas.
Figure 1.8 Example of cancer therapy using Tf attached nanoparticles in vivo. Bartlett et al.\textsuperscript{118} used Tf attached nanoparticles for targeted delivery of therapeutic siRNA. They observed therapeutic efficacy using multimodal in vivo imaging. Figure (A) shows fused micro-PET/CT images showing tumor-associated (arrow) activity 1 day after injection of targeted (Tfn-attached) and nontargeted (PEG-attached) nanoparticles containing $^{64}$Cu-DOTA-siRNA. (B) shows BLI of the same mice shown in (A) before injection and 1 day after injection, and (C) shows the targeted nanoparticles were more efficacious in tumor than their non-targeted counterparts; relative change in luciferase expression 1 day after injection of Tfn-targeted (Tfn, n=7) and nontargeted (PEG, n=4) nanoparticles containing $^{64}$Cu-DOTA-siRNA for simultaneous PET imaging. Error bars indicate SEM. Figure and caption modified from Bartlett et al. 2007.\textsuperscript{118}

Mark Davis et al. have demonstrated successful application of a self-assembled transferrin-PEG-admantane conjugate for the treatment of malignant tumors,\textsuperscript{119} and transferrin (Tfn) conjugated liposomes for the targeted delivery of siRNA in non-human primates.\textsuperscript{120} On the other hand, the Dawson group have recently suggested that Tfn-
conjugated nanoparticles may lose their targeting ability in vivo due to adsorption of serum proteins which leads to formation of a protein corona on nanoparticles. This protein corona can ‘screen’ the targeting molecules on the surface of nanoparticles leading to loss of specificity in targeting.\textsuperscript{121}

Peptides have several advantages over proteins as targeting ligands. The production cost is lower, they are stable over long-term storage and they are more amenable to chemical manipulation.\textsuperscript{106} Unlike antibodies, the risk of an immune response to peptides is lower and, because they are smaller in size, peptides are less likely to change the physicochemical properties of the nanoparticles to which they are attached.\textsuperscript{122,123} Peptides also have a higher activity per unit mass compared with proteins.\textsuperscript{106} The tripeptide arginine-glycine-aspartate (RGD) has been used for targeted drug delivery in many murine tumor models.\textsuperscript{124,125} RGD binds to the cell adhesion integrin $\alpha_v\beta_3$ that is overexpressed on malignant cells and proliferating vascular endothelial cells in tumors.\textsuperscript{126} However, RGD lacks specificity for $\alpha_v\beta_3$; it has been shown to also bind to integrin $\alpha_5\beta_1$ and $\alpha_4\beta_1$, which are expressed by many normal cells, and this has limited the targeting potential of RGD.\textsuperscript{96}

Small molecules, such as folic acid (FA, Vitamin B\textsubscript{9})\textsuperscript{127,128} and biotin (Vitamin B\textsubscript{7}),\textsuperscript{129} have also been widely and successfully employed for targeting cancer cells. The most popular of the small molecule targeting ligands, FA binds with high affinity to the folate receptor-$\alpha$ (FR$\alpha$). FR$\alpha$ has limited distribution on normal cells but is overexpressed by more than a hundred fold on many types of cancers, including ovarian, breast, brain and lung cancers.\textsuperscript{130,131} Wang \textit{et al.} recently utilized FR$\alpha$ targeting to facilitate the cellular uptake of FA-modified, gold-entrapped dendrimer nanoparticles into SPC-A1 cells \textit{in vitro} and human lung adenocarcinoma \textit{in vivo}.\textsuperscript{132} Similar to FA, biotin binds to (strept)avidin with high affinity and this feature is utilized to link (strept)avidin antibody constructs onto biotinylated nanoparticles.\textsuperscript{133} Small molecules are convenient to use as targeting ligands as they are readily available, cost-effective and easy to chemically attach and characterize.\textsuperscript{106} However, they tend to be less selective for binding, and are more susceptible to receptor binding competition from vitamins and other small molecules that are normally present in the diet and body fluids.\textsuperscript{134}
Figure 1.9 Example of higher uptake of FA conjugated nanoparticles, in FR overexpressing KB cells in vitro. Cao et al.\textsuperscript{135} synthesized dendrimer-like star polymers (DLSP) (G1-g3-(COOH)$_{144}$) and conjugated them with FA for targeting and Hilyte-488 for fluorescent imaging (G1-g3-FA-Hilyte488) as shown in the figure (top). To test the targeting capability of these nanoparticles, folate receptor overexpressing KB cells were treated with (A) mock solution (culture medium); (B) G1-g3-Hilyte488; (C) G1-g3-FA-Hilyte488 and free FA; and (D) G1-g3-FA-Hilyte488. Figure shows higher internalization of FA attached conjugates (D) compared with un conjugated nanoparticles (B). However, the addition of free FA in (C) resulted in competitive binding of FA to the receptor, thereby reducing the FA-attached nanoparticle uptake. Overall the results indicate receptor mediated uptake of folate attached DLSP in KB cells. Scale bars - 30 μm. Figure and caption modified from Cao et al. 2010.\textsuperscript{135}
Similar to small molecules, carbohydrates are facile targeting agents. They are readily available, inexpensive, easy to modify and easy to chemically attach to nanoparticles. Carbohydrates may also improve the circulation half-life of nanoparticles by providing them with “stealth” surface properties. Most sugars, such as galactose, lactose and mannose, bind to lectin receptors on cell membrane. Lectin receptors are asialoglycoproteins (ASGPR) which are commonly expressed on liver cells. For this reason, carbohydrate-mediated targeting has been focused on hepatic cancers, although cervical cancer cells have also been tested. Mannose-functionalized mesoporous silica nanoparticles with a covalently attached potent photosensitizer were developed by Gary-Bobo et al. An intravenous administration of these nanoparticles into an HCT-116 colorectal cancer xenograft mouse model followed by photothermal therapy led not only to a reduction of the tumor size but also controlled the formation of micrometastasis in the colon and liver. Another carbohydrate ligand of interest is hyaluronic acid, which targets the CD44 receptor which is expressed on multiple cancer types.

**Aptamers** are novel targeting ligands composed of nucleic acids - DNA or RNA, that bind to their molecular targets. These nucleotide sequences of interest are usually identified by an in vitro screening of a large oligonucleotide library against the desired target molecule. Like the mAbs, aptamers show very high affinity and specificity to their targets. In addition, aptamers are smaller in size and display low immunogenic response. However, for potential utility in vivo, development of enzymatic degradation resistant aptamers is required. Farokhzad et al. developed docetaxel-loaded poly(lactic-co-glycolic acid)-block-poly(ethylene glycol) (PLGA-PEG) nanoparticles to target the prostate specific membrane antigen (PSMA) by covalently conjugating the nanoparticles with an A10 2'-fluoropyramidine RNA aptamer as shown in figure 1. In the LnCaP xenografted nude mice, the free drug, non-targeted nanoparticles and targeted nanoparticles elicited animal survivability of 14%, 57% and 100%, respectively, over a 109 d study period. Mean body weight loss of 7.7% ± 4% and 18% ± 5%, respectively, were observed for the aptamer-conjugated and non-conjugated nanoparticles, with 5 out of 7 animals in the targeted group and 2 out of 7 in the non-targeted group showing complete tumor eradication.
Figure 1.10 Example of active targeting \textit{in vivo} using aptamers conjugated nanoparticles. (A) A10 Aptamer conjugated, dtxl-encapsulated, PEGylated NPs were synthesized using carbodiimide coupling chemistry. (B) The comparative efficacy study of single intratumoral injection (day 0) of (i) saline (black); (ii) PEGylated PLGA NP without drug (NP, brown); (iii) emulsified Dtxl (Dtxl, green), 40 mg/kg; (iv) Dtxl-encapsulated NPs (Dtxl-NP, red), 40 mg/kg; or (v) Dtxl-encapsulated NP-Apt bioconjugates (Dtxl-NP-Apt, blue), 40 mg/kg was evaluated over 109 days and demonstrated that targeted NPs were significantly more efficacious in tumor reduction as compared with other groups. Data represent mean ± SEM of seven mice per group. *, Data points for the Dtxl-NP-Apt group that were statistically significant compared with all other groups by ANOVA at 95% confidence interval. (C) Shows representative mouse at end point for each group is shown (left) alongside images of excised tumors (right). For the Dtxl-NP-Apt group, which achieved complete tumor regression, the scar tissue and underlying skin at the site of injection are shown. Black arrows point to the position of the implanted tumor on each mouse. Figure and caption modified from Farokhzad \textit{et al.} 2006.
1.3 Polymer based nanoparticles as drug delivery vehicles

The general design of polymer based nanoparticles for cancer therapy and imaging comprises of (a) a biocompatible polymeric platform, with or without (b) a targeting moiety and (c) a chemotherapeutic and imaging agent. They can be classified into the following subtypes depending on their design and material assembly.

1.3.1 Polymeric nanoparticles

Polymeric nanoparticles are the most widely used nanoparticles for drug delivery applications. These can be either solid or porous systems with the drug either entrapped, adsorbed, encapsulated or chemically attached to a polymeric matrix. They are described as nanospheres, when the cargo is uniformly dispersed in a polymer matrix, or as nanocapsules, when they are a ‘reservoir’ like system with the drug core surrounded by a polymer membrane (Figure 1.11). The most commonly used synthetic polymers for nanoparticle synthesis are biocompatible and biodegradable polymers like poly(cyanoacrylate) (PCA), poly(lactic acid) (PLA), poly(glycolic acid) (PLG) and poly(lactic-co-glycolic acid) (PLGA). The latter three polymers are FDA approved for biomedical applications. Some natural polymers, such as chitosan, collagen, gelatin, sodium alginate and albumin are also frequently used with or without chemical modifications. Couvreur et al. were some of the first to use PCA nanoparticles. They adsorbed the anticancer drug doxorubicin onto these nanoparticles and demonstrated not only the release of the drug from nanoparticles into fetal bovine serum, but also the tissue distribution and efficacy of the nanoparticles in vivo. This laid the foundation for development of doxorubicin-loaded nanoparticles which later progressed to clinical application in cancer therapy. More recently, polymers such as poly(glycidyl methacrylate) (PGMA) which present large number of reactive functionalities have been successfully employed in nanoparticle synthesis for cancer therapy. Such polymers could provide with the benefit of facile modification with an imaging agent, a targeting ligand and also the drug.

The main concern arising from synthesis of polymeric nanoparticles is the heterogeneity in nanoparticle size, reflected by their high polydispersity index (PDI). However, polymeric nanoparticles offer the advantages of stability and structural rigidity over other nano-delivery systems, which accounts for their popularity. Several polymeric nanoparticles for cancer therapy and imaging are currently in various stages of clinical development.
1.3.2 Polymeric therapeutics

Polymer therapeutics consist of polymers directly and covalently conjugated, usually via a degradable linker, with one or all of the therapeutic, imaging and targeting agents.\textsuperscript{153} The structural basis for most polymer therapeutics is the Ringsdorf model,\textsuperscript{154} primarily consisting of a biocompatible polymeric backbone bound to (1) a solubilizer, which imparts hydrophilicity, (2) a drug, and (3) a targeting moiety.\textsuperscript{154} Polymer therapeutics are amongst the most successful of the first generation of polymeric nanomedicines,\textsuperscript{155} with about 27 products currently approved or in clinical trials.\textsuperscript{153} The majority of these products are being used to target the tumor blood vessels, inhibiting tumors by blocking the nutrient supply. Polymers for fabricating polymer therapeutics include poly(vinyl pyrrolidone) (PVP), poly(vinyl alcohol) (PVA), poly(glutamic acid) (PGA), poly(malic acid), and importantly, PEG and $N$-(2-hydroxypropyl)methacrylamide (HPMA) which are most widely used. The first polymer therapeutic to enter clinical trials was PK1, which consisted of doxorubicin attached to a HPMA copolymer backbone using a lysosomal degradable sequence.\textsuperscript{156} Since then, the versatile HPMA backbone has allowed for conjugation and investigation of a variety of drugs and therapeutic peptides, either alone or in combination.\textsuperscript{157}

1.3.3 Polymeric micelles

Polymeric micelles are sometimes regarded under the category of polymeric therapeutics; however as they differ from polymeric therapeutics with respect to the assembly of composite materials, polymeric micelles are discussed here as a separate category. Polymer micelles are self-assembled structures formed by amphiphilic copolymers dispersed in an aqueous medium. The polymers are usually (1) A-B type copolymer, (2) A-B-A type copolymer, or (3) grafted copolymers with a hydrophilic main chain and hydrophobic branches, or vice-versa.\textsuperscript{158} These polymers assemble to form micelles with a hydrophobic core and a hydrophilic shell in aqueous media. Micelles are excellent carriers for hydrophobic drugs, protecting them from degradation, increasing their aqueous solubility and systemic circulation time.\textsuperscript{159}

Micellar structures are characterized by their critical micelle concentration (CMC), which is the minimum concentration of the uni-mer polymer chains required in a solution for them to assemble into the core-shell micellar structures. CMC values tend to be low ($10^{-7}$–$10^{-4}$ M), and therefore the polymers remain assembled even on dilution \textit{in vivo}.\textsuperscript{160} Another advantage of polymeric micelles is their ease of customization;
targeting moieties can be attached for specific recognition by cancer cells and composite block polymers can be rendered responsive to changes in pH, temperature, light or ultrasound waves for the controlled dissociation and release of the encapsulated drug. PEG is the most commonly used hydrophilic component of the block copolymer because it is water soluble, biocompatible, and has the potential to form a stealth shield for the micelle. Common examples for hydrophobic component include poly(propylene oxide) (PPO), poly(D,L-lactic acid) (PDLLA), PCL, and poly(L-aspartic acid) (PASA). Amongst the block copolymers, the poloxamers (Pluronics®) (PEG-PPO-PEG) are FDA approved and widely used pharmaceutical excipients. Genexol PM is an FDA-approved micellar formulation of paclitaxel encapsulated within PEG-PDLLA micelles for the treatment of recurrent breast cancer. There are about five other polymeric micellar drug formulations presently in clinical trials, of which SP1049C and NK105 are in their final phases.

1.3.4 Liposomes

Liposomes resemble micelles as they are prepared with amphiphilic materials; however the materials used in this case are biocompatible and biodegradable phospholipids that self-assemble into colloidal structures. Liposomes are more versatile than other nanoparticle systems as they can encapsulate both hydrophilic and hydrophobic agents. However, they do have significant limitations, some of which have been resolved through their long history of development. Early liposomes suffered from rapid systemic clearance due to opsonization and sequestration by the reticuloendothelial defense mechanism, with resultant accumulation in the liver and spleen. They also exhibited ‘burst’ drug release and leakage of drug load while in circulation. Significant progress was made following the development of long circulating ‘stealth’ liposomes through PEGylation, remote drug loading, triggered drug release through the addition of agents to the lipid bilayer, and extrusion of the final product to attain homogeneity of carrier characteristics. More than 13 ‘classical’ and ‘stealth’ liposomes are currently in clinical use and many more are in clinical trials. Approved products like DaunoXome® and Myocet® are ‘classical’ liposomal formulations of daunorubicin for the treatment of Kaposi’s sarcoma and doxorubicin for the treatment of breast cancer, respectively. Doxil® (Caelyx™), on the other hand, is the first FDA-approved ‘stealth’ (PEGylated) liposomal formulation of doxorubicin for the treatment of Kaposi’s sarcoma, ovarian cancer and breast cancer.
1.3.5 Dendrimers

Dendrimers are made up of highly branched polymer monomers that radially emerge from a central core. The name comes from the Greek word *dendron* meaning a “tree”, which aptly describes its typical branching structure, with each set of radially concentric layers representing a progressive ‘generation’. The terminal functional groups on the surface of the dendritic architecture are important in influencing the properties of the carrier. Dendrimers are attractive for development into drug delivery systems because they possess the salient properties of monodisperse size distribution, high level of structural control, size similarity with biological entities (e.g. the fifth generation poly(amido amine) dendrimer is globular in shape and about 5.5 nm in diameter, which is similar to the size and shape of hemoglobin), poly-valency, chemically modifiable surface functionalities and capacity for drug loading. However, not all dendrimers are considered to be biodegradable and biocompatible, with toxicity specifically related to the dendrimer concentration, generation and nature of the end group functionalities. Various polymers, both synthetic and natural, have been used to prepare dendrimers. Examples include poly(amido amine) (PAMAM), poly(propylene imine) (PPI), poly(L-lysine) (PLL), triazine, melamine, PEG, poly(glycerol), carbohydrate based citric acid, sugars (glucose, mannose, galactose), disaccharides, peptides etc. Of these, PAMAM is
the most utilized, yielding dendrimers that range from 1.1 nm in size for the first generation (1.0 G) to 9 nm for an 8.0 G dendrimer. A PAMAM dendrimer conjugated to cisplatin displayed significant anti-tumor activity in a subcutaneous metastatic skin melanoma model that was resistant to treatment with free cisplatin. At the maximum tolerable cisplatin dose, the PAMAM formulation was 3- to 15-fold less toxic systemically compared with free cisplatin.

1.4 Preparation of drug-loaded polymeric nanoparticles

1.4.1 Drug loading in nanoparticles

Drug loading into polymer nanoparticles can be achieved by (1) covalent conjugation of the drug to the polymeric backbone, (2) drug adsorption by weak attractive forces to the polymeric nanoparticle surface or (3) physical entrapment of the drug either in the polymer matrix or in the core of a polymeric nanoparticle. Drug entrapment and covalent attachment as shown in figure 1.12 are the most commonly employed techniques.

Figure 1.12 Schematic diagram of drug conjugation and entrapment processes. The chemotherapeutic drug could be chemically bound to the nanoparticle, as in the use of polymer-drug conjugates and drug-conjugated dendrimers (top), or they could be entrapped inside the nanoparticle (bottom). Figure modified from Peer et al.

Covalent conjugation of a drug to a polymer backbone or matrix is an attractive method because a stoichiometric reaction results in reproducible and high drug loading efficiency. It is particularly useful for preparing nanoparticles for combination therapy where a consistent ratio of the two (or more) drugs is to be maintained. However, this method has its challenges. The chemical reactions used must not affect the structural groups that are essential for the pharmacological action of the drug.
molecule. The conjugation bond must be sufficiently labile in vivo so that the parent drug can be regenerated for therapeutic effect. In addition, to minimize the residual toxicity of organometallic catalysts often employed for these chemical reactions, multiple rounds of post-synthesis washing of the product is necessary. The versatility of polymer-drug conjugates was illustrated in a recent example using a water-soluble poly(ethylene glycol)-block-poly(acrylic acid) (PEG-PAA) block copolymer that self-assembled into micelles in aqueous media. Paclitaxel was conjugated to the PAA block via an acid-labile acetal bond to form a prodrug. The final product (paclitaxel-PAA-PEG) was a monodisperse micellar dispersion with high drug loading (up to 42.8% w/w) and pH-dependent drug release profile. The 48 h cumulative paclitaxel release was 86.9%, 66.4% and 29.0% at pH 5.0, 6.0 and 7.4, respectively. The constructs showed high anti-tumor efficacy against Hela and KB cells, and were also effective against paclitaxel-resistant A549 cells. Covalent attachment of FA further enhanced the toxicity of the micelles by 12-fold against the FRα overexpressing KB cells. Combination drug therapy was also possible, as shown by the co-loading of doxorubicin into the micelles and the simultaneous release of the two drugs from the micelles under low pH conditions.

Physical entrapment of drugs in polymeric nanoparticles, on the other hand is a more facile method as it eliminates the need for any chemical reactions and the related concerns regarding reactant toxicity and loss of drug function. For these reasons, despite the lower encapsulation efficiency and poorer reproducibility of drug loading, this method has been the preferred choice for all the nanoparticle-based delivery systems that are currently in various stages of clinical trials. Another reason for its popularity relates to the hydrophobic nature of most chemotherapeutic drugs, since the physical entrapment of the drugs in the nanoparticles can increase their water solubility. In addition, while a burst drug release may be expected from a pH-driven drug cleavage of a polymer-drug conjugate, nanoparticles with physical drug entrapment are dependent on drug diffusion and carrier erosion for drug release, which provides for a sustained release effect suited to chemotherapy. An example is the physical entrapment of paclitaxel in poly(ethylene glycol)-embelin (EB) micelles. This system had a relatively low drug loading of 13.3% w/w, but exhibited a sustained release of paclitaxel over 5 days at pH 7.4, which is considered clinically advantageous. The encapsulated paclitaxel presented with a more favorable safety profile, the maximum tolerated dose (MTD) increased to 100-120 mg/kg compared to 15-20 mg/kg for Taxol®.
physical entrapment methods yield widely variable encapsulation efficiencies depending on the drug, polymer, solvent, temperature, and processing equipment used.\textsuperscript{185,186}

\subsection*{1.4.2 Methods of preparation of nanoparticles with physical drug entrapment}

The preparation method of choice for polymeric nanoparticles with entrapped drug depends on the physicochemical properties of the drug to be encapsulated, the choices of polymer as well as the solvent to be used and the size requirements for the final product.\textsuperscript{183} Conventional synthetic approaches include nanoprecipitation methods, single or double emulsification methods followed by solvent evaporation, diffusion methods and salting out method.

The \textit{nanoprecipitation} method (also known as the solvent displacement or interfacial deposition method) is used when working with two miscible solvent phases (Figure 1.13). Generally, one of them is an organic solvent which is miscible with water and in which both the polymer and the drug are soluble.\textsuperscript{188,189} The organic solution of polymer and drug is added dropwise to the aqueous phase while stirring. Rapid desolvation of the drug-polymer mixture occurs in the non-solvent, leading to their precipitation as nanoparticles which, thermodynamically, favors spherical core-shell structures.\textsuperscript{190} The rapid precipitation is governed by the Marangoni effect, which involves the turbulences created at the solvent-non-solvent interface from the complex interactions of flow, diffusion and surface tension.\textsuperscript{190,191} The particles are harvested once the solvent is evaporated off, often by continuous stirring at atmospheric pressure or reduced pressure, depending on boiling point of the solvent. Nanoprecipitation offers the advantage of a broad choice of organic solvents without the involvement of surfactants, and is amenable to industrial scale-up due to the simplicity of operation.\textsuperscript{192} However, nanoprecipitation yields lower drug entrapment efficiencies, and carries the risk of residual organic solvents.\textsuperscript{193} This technique has mainly been used for the encapsulation of hydrophobic drugs, although it has recently been adapted for hydrophilic drugs via the inverse solvent system, i.e. aqueous into organic precipitation.\textsuperscript{194}
Emulsification techniques involve the formation of an emulsion of two immiscible phases, followed by either the evaporation or diffusion of the solvents to form nanoparticles (Figure 1.14). The emulsification could result in either a single emulsion (oil in water: o/w or water in oil: w/o) or double emulsion (mainly water in oil in water: w/o/w) depending on solvent miscibility in water and drug lipophilicity.\textsuperscript{195}

The single o/w emulsification/solvent evaporation method can be used when the drug is soluble in a water-immiscible phase. Both the polymer and the drug are dissolved in a volatile organic solvent, such as dichloromethane, and added drop-wise into an aqueous phase containing a surfactant, like Pluronic®\textsuperscript{®}, sodium cholate or PVA. This is followed by the introduction of immense shear stress into the system, e.g. homogenization or probe sonication, to form a nanoemulsion. The organic solvent is then allowed to evaporate, resulting in solidification of the polymer and drug into nanoparticles that are stabilized by the surfactant and dispersed in the aqueous phase.\textsuperscript{196} The advantages of the o/w emulsion/solvent evaporation technique are the higher levels of drug encapsulation achievable, and the absence of solvent traces. The nanoparticle size is, however, larger compared to those fabricated by nanoprecipitation.\textsuperscript{196}
The double w/o/w emulsion/solvent evaporation method is used for the encapsulation of hydrophilic drugs, including protein and other hydrophilic biomolecules. The drug is dissolved in a small volume of aqueous phase which is emulsified into an organic solvent containing the polymer. This w/o emulsion is further dispersed into a large volume of aqueous phase with or without a surfactant to form the final w/o/w nanoemulsion, and the organic solvent is evaporated to yield nanoparticles with moderate drug encapsulation and loading efficiencies.

The single o/w emulsification/solvent diffusion method is used for organic solvents which are partially water-soluble, such as benzyl alcohol, propylene carbonate, and ethyl acetate. The polymer and drug are dissolved in the organic solvent which is then emulsified into an aqueous phase containing a stabilizer. A large amount of water is then added to the o/w system to encourage the diffusion of the organic solvent into the aqueous phase, and the solidification of nanoparticles. Cross-flow filtration can be used to eliminate residual organic solvent. This method is suitable for use with hydrophobic drugs and is advantageous over the solvent evaporation method because less toxic solvents, such as benzyl alcohol, can be used to synthesize nanoparticles and the method yields higher entrapment efficiency of drugs. However, downstream processing is required to eliminate a large volume of water from the final nanoparticle dispersion.

The double w/o/w emulsion/solvent diffusion method is used to incorporate hydrophilic drugs whilst employing pharmaceutically more acceptable solvents, like ethyl acetate, and stabilizing agents, like the Pluronic™ surfactants. Milder processing conditions allows this method to be used for encapsulation of proteins, provided the
protein functionality is not adversely affected by the homogenization energy required for emulsification and the generation of the requisite nanoparticle size.\textsuperscript{202} The preparation technique is also advantageous in yielding particles with smaller mean size, narrower size distribution and higher drug entrapment efficiency.\textsuperscript{200}

In the salting out method, a water-miscible organic solvent like acetone is used, but the gross mixing of the solvent with the aqueous phase is prevented by saturating the aqueous phase with electrolytes.\textsuperscript{203} The polymer and drug are dissolved in the organic solvent, and the solution is emulsified into the aqueous phase which, besides the electrolytes, also contains an o/w emulsifier. Water is added to the resultant o/w emulsion to encourage the diffusion of the organic solvent into the aqueous phase, and cross flow filtration can then be used to eliminate both the salts and the residual organic solvent.\textsuperscript{203,204} This method can offer a high drug loading depending on the salts used, and the drug solubility in the organic solvent of choice.\textsuperscript{192} However, it has only been used for lipophilic drugs, and the excessive use of salts can be problematic as the final product needs extensive washing.\textsuperscript{192} Furthermore, some salts may not be compatible with the active compounds.\textsuperscript{192}

More recently, newer approaches have been adapted for the synthesis of drug loaded polymeric nanoparticles which are based on advanced techniques. Most of these methods, like supercritical fluid technology,\textsuperscript{205} electro-hydrodynamic atomization,\textsuperscript{206} premix membrane emulsification,\textsuperscript{207} adopt the same principles as conventional methods, that involve the formation of nanodroplets followed by solvent diffusion or evaporation to precipitate the nanoparticles.\textsuperscript{192} These newer methods are developed mainly with the view to scale up the production of the drug-encapsulating nanoparticles as they offer reproducible batches, and some also allow for the preparation of lyophilized nanopowders.\textsuperscript{192}

1.5 Strategies for the chemical attachment of targeting ligand

Choosing an appropriate chemical coupling strategy for the attachment of a targeting agent to a nanoparticle is important to both ensure nanoparticle stability and ligand functionality.\textsuperscript{208} Surface modifications can impact the physical, chemical and therapeutic efficacy of nanoparticles by changing their size, charge, hydrophobicity and targeting abilities.\textsuperscript{208} It is challenging to choose the right chemistry as even the slightest change or activation of nanoparticles may cause them to precipitate out as agglomerates.
In general, the first step is to check for the availability of functional groups on the targeting ligand and the polymer or the nanoparticle. The next step is to determine if these groups can be used for chemical coupling, bearing in mind the critical functional groups required for molecular recognition of the targeting ligand by its receptor. It is also important to decide if a spacer group needs to be introduced between the targeting ligand and the nanoparticle to optimize the ligand-receptor binding. However, there are no standardized conjugation protocols, and the strategy to employ depends entirely on the nanoparticle, the targeting ligand, and the ligand density and orientation required. The following sections outline commonly used strategies that have been reported in the literature for the chemical conjugation of targeting ligands to nanoparticles.

### 1.5.1 Carbodiimide chemistry

A popular strategy for conjugating molecules onto nanoparticle surface is to utilize the carbodiimide chemistry, where an activated carboxylic acid functionality is reacted with an amine moiety to form an amide crosslink as shown in figure 1.15 A.\(^{106}\) The carboxylate group is activated by reaction with 1-ethyl-3-\((3\text{-dimethylaminopropyl})\) carbodiimide hydrochloride (EDC) or \(N,N'\)-dicyclohexyl carbodiimide (DCC), depending on whether the solvent is aqueous or organic, respectively. This method is popular because of the common availability of amine and carboxylate groups on biomolecules and polymeric chains.\(^{106}\) The reaction can be performed in an aqueous media and the product can be purified by simple dialysis.\(^{209}\) However, the presence of multiple amine or carboxylic acid groups in the reactants can often lead to non-specific, multi-site binding with little control over the ligand orientation.\(^{208,106}\) If a reactant contains both an amine and a carboxylic acid, like certain peptides, they can self-polymerize resulting in loss of efficacy.\(^{208}\) There are also undesired side reactions producing \(N\)-acylurea and rapid hydrolysisation of the \(O\)-acylisourea intermediate.\(^{210}\) To avoid the hydrolysis reaction, an excess of the carbodiimide reagent can be added, but because the intermediate generated in this case is not water-soluble, it can lead to colloidal instability causing aggregation of the nanoparticles.\(^{106,211,212}\) For this reason, \(N\)-hydroxysuccinimide (NHS) is generally added, resulting in an ester intermediate which is comparatively more resistant to hydrolysis. However, the addition of NHS does not resolve the problem of poor control over the conjugation and ligand density that is associated with this strategy.\(^{183,213}\)
Figure 1.15 Strategies for covalent attachment of targeting ligand to nanoparticle. (A) EDC coupling reaction. The 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is a zero-length crosslinking agent used to couple carboxyl groups to primary amines. In the presence of $N$-hydroxysulfosuccinimide (NHS), EDC can be used to convert carboxyl groups to stable amine-reactive NHS esters. The addition of NHS stabilizes the amine-reactive intermediate thus increasing the efficiency of EDC-mediated coupling reactions. (B) Click chemistry reaction. The copper-catalyzed cycloaddition of azides and alkynes (CuAAC) developed for click chemistry joins an organic azide and alkyne together producing a mixture of 1, 4- and 1, 5-triazoles. (C) Maleimide coupling reaction. Maleimide reacts with free sulfhydryl group(s), forming stable thioether linkages at physiological pH.
EDC/DCC-mediated condensation has been utilized to conjugate FA onto polyethylene imine (PEI) coated nanoparticles for targeted gene therapy, and onto iron oxide nanoparticles for targeted imaging applications.\textsuperscript{214,215} To conjugate FA onto a dendrimer comprising of a six-arm star polymer of 2,2-bis(hydroxymethyl)propionic acid,\textsuperscript{216} FA was functionalized with an amino group by carbodiimide reaction with an amine-bearing linker molecule. The amine-functionalized FA was then reacted with the NHS/DCC-activated carboxyl ends of the dendron.

1.5.2 Click Chemistry

Click reactions, also called biorthogonal reactions, involve (3+2) cycloaddition between an alkyne and an azide (Figure 1.15 B). Recognized as a facile and versatile chemistry, these reactions, more specifically the Copper-catalyzed Alkyne Azide cycloaddition reaction (CuAAC) has gained tremendous interest and applications in the field of bioconjugation.\textsuperscript{217} Catalyzed reactions feature a rate acceleration of $10^7$ to $10^8$ compared with the non-catalyzed 1,3 dipolar cycloaddition reactions.\textsuperscript{218} Azides and alkynes are highly energetic functional groups, yet they present with a narrow distribution of reactivity and are inert towards most biological molecules. The azide group can be easily introduced onto an organic compound using nucleophilic or electrophilic processes.\textsuperscript{208} The copper catalyzed reaction between the alkyne and azide then leads to a regioselective 1,4 triazole ring formation.\textsuperscript{22} Keen interest in these reactions stems from their simple approach, high conversion efficiency, high functional selectivity, high reaction specificity, mild reaction conditions, temperature-, solvent- and pH-insensitivity, and generation of only little or no side products.\textsuperscript{106,208,219} This ligation method facilitates control over biomolecule orientation on nanoparticle surfaces.\textsuperscript{220} However, the drawback is that if the azide group is electron deficient, it becomes unreactive. The second problem is alkyne homocoupling.\textsuperscript{221} Homocoupling can be avoided by using a sterically bulky base for the alkyne, for example, having the alkyne on the nanoparticle surface and the azide group on the targeting ligand.\textsuperscript{221} A third drawback is the requirement for the removal of the toxic copper catalyst, either by dialysis or copper ligand and organic scavengers.\textsuperscript{222}

An excellent example that displays the ease of conjugation via click reactions is the synthesis of polymeric micelles that were dual functionalized with targeting antibody and therapeutic peptide via biorthogonal chemistry. Micelles of poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol)-X, where $X =$
azide, were conjugated to dibenzylcyclooctyne-FLAG peptide and maleimide-trastuzumab by sequential click reactions. Co-localization of the antibody and the peptide in SKOV-3 luc cells demonstrated the success of this versatile approach.

1.5.3 Michael addition

Michael addition, in particular the thiol-maleimide coupling, is the second most popular conjugation strategy (Figure 1.15 C). The advantages of this reaction are its selectivity of the sulfhydryl groups to react with maleimide, and greater stability of the 3-thiosuccinimidyl ether linkage relative to the O-acylisourea-nanoparticle intermediate formed by carbodiimide chemistry. Generally, a thiol functionality can be easily introduced on peptides and proteins, including antibodies by reducing an existing disulfide bond (e.g. the disulfide bond in cysteine) or by using heterobifunctional crosslinking agents. It is important to note that, in the absence of a thiol group, the maleimide group will react with amines, which may then cause loss of function for the biomolecules. This requires working in controlled pH conditions (neutral or slightly acidic) to keep the amines protonated. More recently, the utilization of a sulfo-MBS (m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester), which contains both a maleimide and an ester, is preferred for facile attachment of proteins and mAbs. For example, anti-HER2 (trastuzumab) and anti-CD20 (rituximab) were first bound to sulfo-MBS to insert the maleimide functionality before they were reacted with thiolated pyrenebutanol-PLA nanoparticles. In another example, a thiol group was introduced into an A10 RNA aptamer, which targets prostate cancer cells, by oxidation of a hydroxyl group from the 5’-end GTP into an aldehyde followed by reaction with the free amine group of cystamine. Nanoparticles were prepared from PLGA and PEGylated DSPE, the PEGylated tail of the lecithin functionalized with maleimide for attachment of the thiolated RNA. The thiol-maleimide conjugation method allows the control of ligand orientation, and maintains ligand structural properties to optimize targeting. Its main limitation is the hydrolysis of the maleimide ring to a non-reactive cis-maleamic acid over long periods of reaction or at pH greater than 8.0.

1.5.4 Non-covalent strategies: Ionic coupling and biotin-avidin ligation strategy

Ionic coupling is the direct coupling of two oppositely charged entities and has been widely used for the adsorption of charged biomolecules like nucleotides, peptides and proteins onto nanoparticles (Figure 1.16). The rate of coupling depends on the nature
and amount of charge present on both the nanoparticles and the targeting moiety. This can be determined from their isoelectric point and zeta potentials. Cationic and anionic nanoparticles have been well researched for nucleic acid and peptide attachment followed by their nanoparticle-aided intracellular delivery. A PAMAM dendritic platform for gene delivery was developed based on the ionic coupling of its cationic surface with the anionic phosphate groups of nucleic acid. On the other hand, Han et al. functionalized a PAMAM dendrimer with hyaluronic acid, and used it to ionically bind siRNA for silencing the expression of the major vault protein in the drug resistant-MCF-7/ADR cells. It is generally considered that cationic groups on dendrimer surfaces determine the nanoparticle efficiency for ligand attachment and transfection. However, Kwok et al. in a recent evaluation of dendrimers with different cationic and hydrophobic amino acid motifs for transfection suggested that the successive placement of cationic charge distribution in the inner shells (rather than just the outer surface) could significantly increase transfection capacity. Despite the ease of attachment ionic coupling offers, the native structure and conformation of adsorbed proteins can be affected, resulting in loss of biological activity. Electrostatic interactions are pH dependent, so a change in the ionic strength or pH may incur desorption of the ligands from the nanoparticle.

By comparison, the other non-covalent binding method, biotin-avidin ligation strategy is a more attractive method by virtue of its flexibility and strong adhesion (dissociation constant, $K_d = 4 \times 10^{-14}$ M). Biotinylation of molecules and nanoparticle surfaces is very commonly practiced, and the biotin molecule can be synthesized to have a distal amine, thiol, carboxyl or other functional group for further conjugation. Genetic engineering has further facilitated the commercial availability of recombinant biotinylated antibodies and biomolecules. Avidin, a zwitterionic molecule, is usually electrostatically adsorbed onto nanoparticles to allow for binding of the nanoparticles with a biotinylated targeting ligands. However, avidin is a positively charged glycoprotein (isolectric point $\sim$10), and it is prone to unspecific binding of various biomolecules. For this reason, streptavidin, a non-glycoprotein with $pI \sim 5-6$ is preferred. Streptavidin binds with high affinity like avidin, but both avidin and streptavidin are tetrameric in nature. Therefore, when stoichiometry is important, low affinity recombinant monomeric forms of avidin or streptavidin are used.
Figure 1.16 Ionic coupling of targeting ligand. Coupling of negatively charged siRNA to positively charged nanoparticle (left) and of positively charged peptides to negatively charged nanoparticle (right).

Townsend et al. compared the efficiency of biotin-binding avidin, streptavidin and neutravidin to conjugate proteins to biotinylated PLGA-PEG nanoparticles. They found avidin to give the highest conjugation rates, but neutravidin minimized the non-specific binding of the protein to the polymer. Park et al. coated PLGA nanoparticles with an avidin-palmitate bioconjugate in order to bind a biotinylated anti-CD4 antibody to target T cells for leukemia therapy. Zhou et al. used a similar strategy to attach three different biotinylated peptides onto an octa-functional nanoparticle to facilitate cell penetration, endosomal escape and tumor targeting.

1.6 Multifunctional nanoparticles

Recent advancements in the field of nanomedicine, has resulted in successful application of a number of nanoparticle-based therapeutics and imaging agents in the clinic. However, these clinically used nanoparticles have the downside of performing only one primary function at a time, when nanotechnology holds the potential of integrating two or more agents into a single delivery system. Recognition of this potential has led to immense research interest in fabrication of multifunctional nanoparticles which aim to achieve a combination of features as presented in figure 1.17, including (1) ‘stealth’ covering to prolong systemic circulation; (2) protective coating to minimize cargo degradation; (3) targeting moiety to direct and enhance delivery to a specific organ and cell; (4) membrane permeation functionality for assisted and preferential translocation into cells; (5) endosomal escape mechanisms to facilitate intracellular drug release; (6) imaging agents for diagnosis and simultaneous assessment of therapeutic efficacy; (7) target-dependent assembly or disassembly of the delivery
system for on-demand drug release; and (8) sensor to trigger cellular uptake and drug release.

Figure 1.17 Multifunctional nanoparticles for drug delivery. Ideal multifunctional nanoparticles can combine a specific targeting agent, an imaging agent (such as quantum dots or magnetic nanoparticles), a cell-penetrating agent (e.g. the poly(arginine) peptide - TAT), a stimulus-sensitive element for drug release, a stabilizing polymer to ensure biocompatibility and the therapeutic compound(s). Figure and caption from Sanvicens and Marco 2008.

An excellent example of a multifunctional nanoparticle system has recently been successfully tested in Phase 1 clinical trials for treatment of metastatic melanoma. CALAA-01 is a sub-100 nm siRNA delivery system utilizing a cyclodextrin carrier which has been rendered cationic by amino functionalization to enable it to form an ionic complex with the anionic siRNA. To minimize opsonization and systemic clearance on account of the net positive charge, PEGylation was introduced by the formation of a host-guest complex between a PEG chain with a hydrophobic terminal group and the cyclodextrin core. Targeting capability was imparted to this construct by the chemical conjugation of transferrin (Tfn) to the extending PEG chain. Compared to the non-targeted construct, CALAA-01 showed a doubled knockdown effect on tumor gene expression.

Reddy et al. have also reported significant enhancement in the magnitude and duration of tumor contrast imaging using polyacrylamide-based multifunctional nanoparticles prepared using iron oxide nanoparticles as imaging agent and Photofrin as a sensitizer for photothermal therapy. The nanoparticles were further PEGylated to prolong systemic circulation, and an F3 peptide was attached for active targeting to tumor...
vasculature.\textsuperscript{249} MRI revealed nearly 3-fold increase in tumor residence half-life of the nanoparticles and significantly higher mean tumor apparent diffusion coefficient compared to non-targeted particles and free sensitizer in glioma-bearing rats.\textsuperscript{249}

Unlike few of these successful examples, the majority of the multifunctional nanoparticles are in pre-clinical development stages. One of the hurdles in the realization of their clinical outcome is the increased cost associated with the synthesis and evaluation of these complex systems. The addition of each new functionality, while desirable for its beneficial impact on therapeutic and diagnostic outcome, contributes to additional regulatory, production, and evaluation hurdles. The cost to benefit ratio will depend on the choices made when designing the multifunctional nanoparticles, as represented in figure 1.18.
Figure 1.18 Multifunctional nanoparticles: Cost versus benefit of adding targeting and imaging capability. Multifunctional nanoparticles can be prepared with a wide range of therapeutic, imaging, and targeting agents. Representative examples are shown, with relative expenses indicated by dollar symbols: Targeting (left: folic acid, center: antibody, right: aptamer); Imaging (top: chelated Technetium-99m, center: chelated gadolinium, bottom: near infrared fluorescent dye-isocyanine green); Therapy (left: doxorubicin, center: paclitaxel, right: camptothecin) Figure and caption from Cheng et al.250

1.6.1 Multimodal Imaging: The possibility and advantages of using more than one imaging agent incorporated into a single nanoparticle

Continuous progress in the field of imaging technology has provided us with a better understanding of the heterogeneous nature of tumor tissue. It has also empowered us with the capacity to optimize anticancer therapy based on more accurate diagnosis and better precision in the visualization of the target site. Imaging modalities that are currently being used in the clinic include magnetic resonance imaging (MRI), computed tomography (CT), ultrasound (US), positron emission tomography (PET) and single photon emission computed tomography (SPECT).251 Each of the imaging techniques
have their own advantages and limitations, which is why utilization of a combination of complementary imaging modalities has gained attention. The first instrument for combined PET/CT imaging was commercialized in 2001 and since then, a large number of nanoparticle-based probes for multimodal imaging have been developed.\textsuperscript{252} Nanoparticles can offer the co-delivery of two or more imaging agents, as well as the capacity for targeted delivery of these agents to the tumor site.

Imaging methods are usually combined for their synergism, e.g. magnetic resonance imaging (MRI) with its high spatial resolution is combined with optical fluorescence imaging for its high sensitivity. MRI is a highly valued diagnostic tool that can generate 3D images from differences in proton density and relaxation time to differentiate the anatomical contrasts of opaque and soft tissues,\textsuperscript{253-255} but MRI has inherently low sensitivity.\textsuperscript{256-258} Optical imaging techniques, such as confocal microscopy, fluorescence microscopy and \textit{in vivo} intravital microscopy, are complementary to MRI\textsuperscript{259} as they allow for the detection of multiple fluorescent tags at high speed and sensitivity. Conventionally applied \textit{ex vivo}, the availability of new technologies has now made optical imaging a reality \textit{in vivo}\textsuperscript{260} However, optical techniques have limited depth of tissue penetration, and consequently poor resolution of anatomical information. For this reason, a combination of MRI and optical techniques could be advantageous in providing more precise and detailed imaging, and this is accessible by developing nanoparticles tagged with both fluorescent and magnetic resonance contrast agents.\textsuperscript{261} Novel MRI contrast agents, such as gadolinium nanoparticles and iron oxide nanoparticles, have high relaxivities and have become popular for MRI applications.\textsuperscript{262,263}

An exemplary multimodal imaging system is reported by Rolfe \textit{et al.}, wherein they attached trifluoroethyl acrylate and rhodamine B dye-NIR797 dye onto a hyper-branched PEG scaffold for $^{19}$F MRI and multispectral fluorescence imaging, respectively.\textsuperscript{264} This combination enabled the researchers to visualize tumor mass on a scale that ranged from millimeters to as low as tens of micrometers.\textsuperscript{264} While both imaging techniques clearly showed major organ accommodation of the system after an \textit{i.v.} injection, the fluorescence images provided whole animal tracking of the nanoparticles, while the MR images complemented high resolution images of particle distribution in individual organs.
In another report, Dumont et al. synthesized a core-shell nanoparticle with the core consisting of a Prussian blue lattice with gadolinium ions located in its interstices. This conferred the nanoparticles with high relaxivity for use as an MRI contrast agent. The nanoparticles were further biofunctionalized with fluorescently labelled avidin for confocal imaging, and with biotinylated antibodies for specificity of delivery. This multimodal construct enabled the specific detection of eosinophilic cells within a mixed blood cell culture and provided scope for progress into in vivo testing.

1.7 Assessing nanotoxicity

In conventional terms, material toxicity is governed by only two parameters, concentration of the toxin and the exposure time. For a chemical with known dose-response profile, a threshold concentration level exists which helps to determine if the chemical entity at a particular level is ‘safe’ or ‘dangerous’. ‘Nanotoxicity’ on the other hand is a relatively complex term as it relates to the toxicology of materials at the nanoscale. This implies, nanotoxicity is a result of physical and chemical properties of materials which are not present at either molecular or in its bulk forms. In general, nanoparticles exhibit greater cytotoxicity than the corresponding bulk materials because of their higher surface reactivity, and their access to size-restricted interactions at the cellular level. The complexity of such interactions makes it difficult to clearly define pathways and mechanisms, and to accurately predict the toxic effects of a nanoparticle. The level of toxicity is dependent on the nanoparticle chemistry, dose, exposure duration, route of administration (oral, inhaled, topical), and organs of accumulation (lung, liver, heart and brain). In addition, toxicity can also be influenced by the nanoparticle size, shape, surface chemistry, surface charge, aggregation status, and morphology.

The production of novel nanomaterials and their application have grown exponentially but the protocols for assessing their toxicity with respect to human health have developed rather slowly. Toxicological evaluations are routinely conducted in vitro in 2D monocultures of cancer or immortalized cell lines. The preferential use of primary cells and stem cells for toxicity studies is increasing because these make better representative test cells. The selection of relevant cell types depends on the expected distribution in organs in vivo. In majority of the published studies, selected cells are exposed to the nanoparticle dispersions during a single period, which may range from several hours to a few days. The cytotoxicity induced over time is then determined by
one or more of the biochemical assays, such as the Live/Dead® assay, tetrazolium based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay,\textsuperscript{274,275} which evaluate cell viability or estimate live cell numbers by measuring the extent of a cellular metabolic activity. These assays are of short duration, uncomplicated and automatically executable for fluorometric or spectrophotometric read-outs. At the cellular level, a number of markers for toxicological damage have been identified, including reactive oxygen species (ROS), misfolded proteins, membrane perturbations and direct physical damage (Figure 1.19).\textsuperscript{266}

![Figure 1.19 Mechanisms associated with nanoparticle toxicity at the cellular level.](image)

While the \textit{in vitro} cytotoxicity data is helpful in assessing nanoparticle toxicity, \textit{in vitro} assays are unable to replicate the dynamic and complex \textit{in vivo} environment.\textsuperscript{276} Most \textit{in vitro} experiments are reliant on single cell lines which makes them unable to reproduce the intercellular communication between different cell phenotypes that exists \textit{in vivo}.\textsuperscript{277}
Secondly, 2D in vitro cell cultures do not reproduce the spatial structure and limitations of a 3D tissues which has a complex makeup of different cell types, membrane polarization and extracellular matrix (ECM).\textsuperscript{278} The ECM composition plays an important regulatory role in homeostasis and phenotypic expression in vivo and this is not readily reproducible by the cell culture media employed in vitro. On the other hand, an altered and incomplete extent of ECM possibly makes nanoparticle diffusion easier in vitro.\textsuperscript{279,280} Finally, the fluid dynamics in vivo can not be replicated in the culture vessels, where sedimentation of nanoparticles onto the cell surface can influence their toxicity profile. Therefore, in vivo toxicology testing is required. In vivo evaluations involve the exposure of selected animals to test doses of nanoparticles that are derived from the in vitro cytotoxicity experiments.\textsuperscript{281} Despite being more realistic in toxicity evaluation, in vivo studies are limited by high cost and also invite ethical criticism.\textsuperscript{282,283}

However, in recent years, there is a move towards the use of 3D cell cultures to provide a simulation of the in vivo environment and consequently yield data of higher predictive potential. Lee et al.\textsuperscript{284} evaluated the toxicities of cadmium-telluride quantum dots (CdTe QDs) and gold nanoparticles (Au NPs) using 3D spheroid cultures of HepG2 cells.

Physiologically relevant and standardized 3D liver tissue spheroid model for in vitro assay application were prepared using polyacrylamide hydrogel inverted colloidal crystal (ICC) scaffold.\textsuperscript{284} The structure of an ICC scaffold primarily consists of organized and uniformly sized spherical pores for standardization of spheroid diameter (and total cell numbers).\textsuperscript{285} This is critical as spheroids with excessively small diameters may not have physiological properties of a tissue level while large diameters can cause cells at the centre of the spheroid to suffer from hypoxia and an inadequate nutrient supply.\textsuperscript{286,287} Polyacrylamide was used for its biocompatibility, transparency and nonfouling properties to create a cell-repulsive hydrogel matrix with favourable physicochemical characteristics for formation of spheroids with a narrow size distribution while the high optical analytic capability is maintained.\textsuperscript{288} In this work, Lee et al.\textsuperscript{284} modified their original ICC design to have open pores only at the top for cell seeding while the bottom and edges remained enclosed to avoid cell loss. To control the spheroid diameters to about 100 µm, glass beads with approximately 156 µm diameter were used for colloidal crystal (pore) formation, forming a scaffold with about 174 µm in pore size and 50 µm interconnecting channels. The hydrogel scaffold was inoculated with a small suspension of dense cell suspension (5 x 10^5 Cells per 20 – 30 µl) following up with addition of 1 ml culture medium. Half a volume of the culture
medium was changed daily. After 3 to 5 days of culturing, individual cells formed solid spheroids of the required diameter.\textsuperscript{284}

On spheroid formation, both 2D cell cultures and spheroids were exposed to nanoparticles at a fixed concentration for 12 or 24 h followed by an MTT assay. Nanoparticle toxicity studies indicated that relative to the 2D cultures, the spheroid cultures were more resilient to nanoparticles. Prolongation of the exposure times led to increased cell death for the 2D cultures but for the 3D spheroids, only cells in the periphery were affected while cells in the interior core of the spheroid remained unimpaired. The authors attributed this difference in toxicity profiles of nanoparticles in 2D and 3D cultures to the protective barrier effect of the ECM in the 3D spheroid cultures.\textsuperscript{284}

1.8 Summary of literature and thesis rationale

Intensive research over the past decades has led to significant advances in diagnosis and treatment of cancer, yet global statistics suggest that presently available imaging and therapeutic agents are still far from optimal. To date, chemotherapy remains a mainstay treatment choice, but the non-discriminative action of most anticancer drugs limits the maximal tolerable dose of a drug that can be administered to a patient. Of the administered dose, often only a sub-optimal amount is accumulated at the tumor site leading to only moderate efficacy.

Nanoparticle-based therapeutics have clinically demonstrated the potential to overcome the aforementioned limitations of conventional chemotherapy. A variety of nanoparticles have been reported to improve the therapeutic index (TI) of potent anticancer drugs by the mechanism of passive accumulation in tumor interstitium. This improvement in the drug pharmacokinetic profiles has been associated with fewer systemic effects. However, it is not often concomitant with significant enhancement of drug efficacy. This has provided the impetus for the development of strategically designed multifunctional nanoparticles, with most preliminary and pre-clinical data suggesting improved retention in tumor attributable to a more prolific uptake by the target cancer cells. The capacity of these nanoparticles to carry multiple payloads, i.e. a combination of drug(s), imaging and targeting agents, would provide longitudinal information about drug localization and tissue pathological resolution, while minimizing the limitations of conventional chemotherapy such as the development of MDR in the target cell.
Despite their potential, there are significant cost barriers associated with the production, evaluation and regulatory approval of multifunctional nanoparticles. Each additional component requires an additional synthesis pathway or a fabrication process along with testing to ensure safety, quality and efficacy compliance. To reduce cost, it is paramount to adopt a quality-by-design concept at the outset with an intelligent choice of materials, synthesis technique, conjugation methods and evaluation methodologies.

For the synthesis of multifunctional nanoparticles as described in this thesis, the choice of the drug was determined first as it influences the choice of other materials and methodologies to be used for the assembly of the carrier platform. Docetaxel is a potent FDA-approved drug for application in treatment of ovarian and prostate cancers. It was chosen for nanoparticulate encapsulation because a reformulation of this drug could be beneficial to overcome the adverse reactions associated with its clinical formulation, Taxotere®. The high incidence of systemic toxic effects associated with Taxotere® are dose limiting. Moreover, docetaxel treatment has been reported to result in the development of MDR.

It was preferred to use a polymeric material for nanoparticle preparation due to their structural rigidity, integrity and versatility, and their capacity to allow for cargo entrapment and attachment, as well as protection from degradation in vivo. Features such as biocompatibility, resistivity to degradation to enable sustained drug release, low cost and most importantly, ease of functionalization, were important considerations for this study. Poly(glycidyl methacrylate) (PGMA) was the polymer of choice because of its previously demonstrated biocompatibility. PGMA also presented with the epoxide functionality, which could be well suited for highly efficient attachment of both the imaging fluorophore as well as the targeting ligand via epoxide ring opening reactions.

The nanoparticle platform was designed for multimodal imaging by the incorporation of complementary imaging agents. Rhodamine-B (RhB) (Figure 1.20), a laser-tunable fluorophore, could be covalently attached to PGMA, via its carboxylic group utilizing the epoxide ring-opening reaction, to facilitate microscopic visualization of the nanoparticles in cell cultures. However, like most organic fluorophores, RhB has poor signal to noise ratio in vivo due to background interference by tissue auto-fluorescence. Therefore, magnetite nanoparticles, a complementary T2 contrast generating MRI agent, were loaded to assist in the biodistribution assessment of the nanoparticles in vivo.
Facile synthesis of oleic-acid coated superparamagnetic iron oxide nanoparticles is feasible by well-established methods.

Figure 1.20 Rhodamine B. Chemical structure of Rhodamine B (inset) and its absorption and emission spectrum.

The delivery platform was designed to target ovarian cancer (represented by the SKOV-3 cell line in this thesis) and castration-resistant prostate cancer (represented by the PC3 cell line in this thesis). Over 90% of ovarian cancers have been shown to overexpress the folate receptor-α, therefore either FA or an antibody against the folate receptor would be an appropriate targeting ligand for selective receptor mediated uptake of the nanoparticles in ovarian cancer cells. Considering the cost, availability and stability of the ligands, FA was preferred. The PC3 prostate cancer cell line is deficient for most signature prostate cancer cell markers, but these cells overexpress the transferrin receptor, therefore the protein transferrin was selected as the ligand for specific receptor mediated uptake of nanoparticles in these cells.

Given the hydrophobic nature of PGMA, docetaxel, and iron oxide nanoparticles, an oil in water (O/W) single emulsion technique was selected for the synthesis of the nanoparticles with entrapped docetaxel and iron oxide nanoparticles. Transferrin could be easily attached to the PGMA nanoparticle surface via a nucleophilic ring opening reaction between the PGMA epoxide groups and a primary amine on the protein. On the other hand, FA attachment was not feasible via a simple one step ring opening reaction, but the attachment of the distal glutamate moiety of FA onto the polymer backbone was accessible via the widely used carbodiimide (NHS/DCC) mediated chemistry. However, the ‘click’ reactions are preferable to the carbodiimide methods for their shorter reaction times, greater assurance of nanoparticle stability, control of ligand density, and benign
reaction conditions, together with negligible downstream purification requirements. This would require the development of a novel ‘click’ facilitated conjugation methodology for the attachment of FA onto the PGMA nanoparticles.

The specific objectives of the thesis were:

(1) To synthesize and characterize FA-attached, RhB labelled PGMA nanoparticles with the hypothesis that these nanoparticles would be biocompatible and would show higher uptake in FR-overexpressing ovarian cancer cells (SKOV-3 human ovarian cancer cells) in comparison with FR-deficient cells (A549 human lung adenocarcinoma cells); and

(2) To synthesize and characterize transferrin-tethered, RhB labelled PGMA nanoparticles loaded with docetaxel and magnetite nanoparticles. It was hypothesized that these multifunctional nanoparticles would demonstrate better efficacy and less toxicity than the non-transferrin tethered nanoformulation and the clinical docetaxel formulation in an orthotopic mice model (PC-3) of prostate cancer. It was also hypothesized that these nanoparticles would not only allow for the targeted delivery to the PC-3 tumor, but also allow for the assessment of \textit{ex vivo} nanoparticle distribution using proton transverse relaxometry.

Chapters 2 and 3 of the thesis describe the experimental methodology adopted and present a discussion of the results obtained for the aforementioned objectives respectively. Chapter 4 concludes the thesis with a discussion of the findings of the current work, possibility of its future improvement and thoughts in the direction of future extension of multifunctional nanoparticles synthesized for work presented in this thesis.
Chapter 2

Poly(glycidyl methacrylate) (PGMA) Nanoparticles as Versatile Platforms for ‘Click’ Assisted Folate Functionalization and Receptor Targeting

2.1 Folic Acid and folate transporter proteins

Folic acid (FA) or folate (pteroylglutamic acid or pteroglutamate) which is commonly known as vitamin B9 along with its derivatives are essential nutrient supplements that have a central role in DNA synthesis, repair, and methylation. They are important in aiding rapid cell division and growth, especially in infancy and pregnancy. Folate transport into cells and across the epithelia occurs by one of the two possible mechanisms: (1) Carrier-mediated transport or (2) Receptor-mediated transport. Carrier-mediated folate transport occurs via membrane spanning proteins belonging to the superfamily of solute carriers (SLC); (a) The Reduced Folate Carrier (RFC; SLC19A1) and (b) The Proton-Coupled Folate Transporter (PCFT; SLC46A1), while receptor-mediated folate transport occurs via the Folate Receptors (FRs) which are expressed on the cell surface and anchored to the membrane by means of glycoprophosphatidylinositol (GPI).

The transport kinetics for carrier-mediated transport of folate are described by Michaelis-Menten kinetics, with the binding affinity expressed using Michaelis-Menten constant, $K_m$. As $K_m$ indicates the concentration of substrate at half the maximum influx rate; a lower $K_m$ means higher affinity of the receptor to the respective folate. The RFC is ubiquitously expressed on all cell types as it is a major transporter of folates in systemic tissues, with an influx that is optimal at pH 7.4. It has a high micromolar affinity ($K_m \sim 2-7 \mu M$) for reduced folates (5-methyl tetrahydrofolate, 5-formyl tetrahydrofolate) as well as antifolates (methotrexate, pralatrexate, pemetrexed etc.), but very low micromolar affinity for FA ($K_m \sim 200 \mu M$). PCFT on the other hand, has high micromolar affinity for both reduced folates and FA ($K_m \sim 1-5 \mu M$) and the influx...
is optimal in low pH conditions (~ pH 5.5).\textsuperscript{285,300} PCFT is suggested to have low expression in the brain, testis and lungs, but high expression in the kidneys, liver, placenta, spleen and, importantly, the intestines, where it is responsible for the absorption of exogenous folates.\textsuperscript{287,301}

The binding affinity of FRs which internalize folates by receptor mediated endocytosis is described using dissociation kinetics and expressed using the dissociation constant $K_d$. The FRs have high nanomolar affinity ($K_d < 1-10 \text{ nM}$) for FA at its physiological concentrations (pH 7.4).\textsuperscript{302} FRs are expressed in three isoforms - FRα, FRβ and FRγ (encoded by the genes FOLR1, FOLR2 and FOLR3 respectively) with each of the isoforms having its own tissue specific expression, function and biochemical properties.\textsuperscript{303,304} While FRγ is a secretory protein predominantly expressed in hematopoietic cells, both FRα and FRβ are membrane-bound GPI-anchored proteins that are expressed at high levels in diseased cells.\textsuperscript{130,305}

### 2.2 Folate Receptor-α (FRα) is overexpressed in cancer cells and FA is an ideal ligand for nanoparticle-mediated cancer targeting

In cancer, receptor overexpression is a direct result of the higher need of folate by rapidly dividing cells for cellular metabolism, nucleic acid synthesis and repair.\textsuperscript{306} FRs are of particular interest for targeted therapeutic delivery due to their overexpression in cancer cells, limited systemic expression and importantly their role in the endocytic mode of cellular internalization. Out of the three aforementioned isoforms, FRα is the most widely studied and found to be mainly expressed on the placenta, the choroid plexus, apical brush-border membrane of proximal renal tubular cells and retinal pigment epithelium. However, FRα is overexpressed in various types of human cancers, especially cancers of epithelial lineage like the ovarian, endometrial, colorectal, breast, lung, renal cell carcinoma, brain and neuroendocrine carcinomas.\textsuperscript{307,308} In particular, nearly 90% of ovarian carcinomas overexpress FRα.\textsuperscript{130,309-311} The levels of FRα expression are also suggested to be positively associated with tumor stage, aggressiveness and grade, which indicates a possibility of its role in tumor progression.\textsuperscript{307} FR targeted therapeutics have been widely investigated and they utilize targeting ligands such as monoclonal antibodies to FR, folate-conjugates and anti-folates.\textsuperscript{312,313} Out of a number of these ligands available for FR-targeted anticancer therapy, FA is a molecule with high storage stability, ease of chemical manipulation and
characterization, readily available and cost effective, making it a ligand of choice for conjugation to nanoparticles for targeted theranostic applications.\textsuperscript{127}

2.3 FA conjugation to nanoparticles

2.3.1 FA should be conjugated \textit{via} its glutamate moiety for molecular recognition by FRα

It is important to understand the structural basis for molecular recognition of FA by FRs in order to optimize the chemistry and orientation for attachment of FA as a ligand on nanoparticles for targeted delivery. Recently, the determination of the crystal structure of human FRα in complexation with FA\textsuperscript{314} (Figure 2.1) has suggested that the ptereoate moiety of FA is buried inside the receptor while the glutamate moiety is free, which makes the carboxylate groups the optimal region for conjugation of FA to nanoparticles without affecting its receptor affinity. Although this has only been substantiated recently\textsuperscript{314}, FA conjugation to nanoparticles has, with few exceptions, mostly been practiced \textit{via} the carboxylate group.\textsuperscript{315} Out of the two carboxylate groups present on FA, Leamon \textit{et al.} have suggested that attachment \textit{via} either the αGlu or the γGlu does not provide for any selective endocytic advantage over the other.\textsuperscript{316}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Structural orientation of FA in association with FRα: (A) Molecular structure of FA. (B) Structure of FRα bound to FA with a close up view of FA in the form of a 3D stick structure with carbon atoms colored grey, nitrogen atoms are blue, and oxygen atoms are red. (C) An internal view of the binding pocket and FA in stick configuration. Alphanumeric symbols in (B) and (C) represent amino acid residues as mentioned in the reference paper. Figure adapted from Chen \textit{et al.} 2013.\textsuperscript{314}}
\end{figure}
2.3.2 Carbodiimide chemistry is most commonly used, but not the most optimal methodology for FA conjugation

Polymers poly(cyanoacrylate) (PCA), poly(lactic acid) (PLA), poly(glycolic acid) (PLG) and poly(lactic-co-glycolic acid) (PLGA) are the most commonly used polymers for nanoparticle synthesis mainly due to their biocompatibility, biodegradability and FDA approval of the latter three.\textsuperscript{186} Furthermore, conjugation of PEG to the polymers or nanoparticle surface is also a common practice to improve the biodistribution and circulation half-life of these nanoparticles.\textsuperscript{317} In all of these cases, the resulting polymer or nanoparticles have either a carboxylic acid or a hydroxyl functionality present on them which can be easily converted to an amine. For this reason, the \textit{carbodiimide chemistry} (NHS/DCC; \textit{N}-hydroxy-succinamide with \textit{N},\textit{N}'-dicyclohexyl-carbodiimide or NHS/EDC; \textit{N}-hydroxy-succinamide with 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) involving a carbodiimide and \textit{N}-hydroxy succinamide in order to activate the carboxylic acid, and then enable a cross-linking reaction with an amine on the nanoparticle surface to give a stable amide linkage, is the most utilized methodology for FA conjugation.\textsuperscript{215,216,318-334} Carbodiimide-assisted attachment has been a convenient chemical strategy for FA attachment, but it is not the most optimal method as it suffers from drawbacks which include: possible nanoparticle instability and aggregation on intermediate formation (as it is not water soluble), susceptibility to intermediate ester hydrolysis and other factors causing low conjugation efficiency and little control on ligand density.\textsuperscript{106,213} The final product obtained also requires purification from excess NHS, by-products and solvents, which may present considerable challenges.
2.3.3 “Click” chemistry approach is more efficient and cleaner than traditional FA conjugation approaches

Click chemistry has had a large impact on efficient functionalization of nanoparticles using a variety of targeting ligands. The philosophy underpinning Sharpless and co-workers’ discovery of ‘click’ chemistry is to restrict all searches to the synthesis of molecules that are easy to make. The approach was to follow nature’s lead, which is to generate substances by joining smaller units together with heteroatom links (C-X-C) (Figure 2.2). For this reason, they developed a set of “powerful, selective and modular blocks that work reliably in small- and large-scale-applications” and this is why the approach was termed “click chemistry”. Click reactions have a stringent definition, they must be high yielding, stereospecific, modular, wide in scope and generate inoffensive by-products that can be easily removed by non-chromatographic methods. Furthermore, the process must use simple reaction conditions, readily available starting materials and reagents, and a solvent-less approach or benign solvents most preferably water.

Several types of reactions have been identified that fulfil these criteria, and they are classified as (1) Cycloaddition reactions, (2) Nucleophilic ring-opening reactions, (3) Non-aldol carbonyl chemistry and 4.) Carbonyl multiple bond additions, (Figure 2.2).

Among these reactions, the Copper (I) catalyzed Azide-Alkyne Cycloaddition reaction (CuAAC) to form 1,4-disubstituted triazoles is the most widely used. As it does not require the use of high temperature, varied pH or volatile organic solvent systems, click chemistry is well suited for the facile attachment of sensitive biomolecules as targeting ligands onto nanoparticles.
2.3.4 “Click” chemistry for ligand conjugation results in a multi-step process

It should however be noted that the adoption of click chemistry for bioconjugation reactions results in a multi-step process, requiring pre-functionalization of the starting materials. For example, for the preparation of FA-conjugated nanoparticles via the click route, Saeed et al. first reacted the block copolymer, PLGA-S-PEGMA475, with sodium azide over 24 h at 50 °C in DMF, and then removed the insoluble side products to obtain an azide-functionalized polymer. An alkyne group was introduced on FA by first activating the glutamate moiety using NHS/EDC in water, followed by reaction with propargylamine over 24 h at room temperature in DMF. Finally, copper-catalyzed “click” reaction of the azide functionalized PLGA-S-PEGMA475 block copolymer with propargylamine functionalized folate was conducted under argon, in DMF over 24 h to yield the folate terminated polymer for further preparation of nanoparticles by w/o/w emulsification. These multiple steps, to some extent defeat the purpose of adopting a simplistic click approach.
2.4 PGMA nanoparticles for simplified ‘click’ assisted FA conjugation

The presence of a large number of reactive epoxide functionalities (one epoxide per monomeric unit) makes PGMA a very versatile polymer, which is ideal for both the synthesis of nanoparticles and their facile modification. In this chapter, PGMA nanoparticles are presented as platforms for the efficient attachment of FA as a targeting ligand via two different, consecutive click reactions. Herein, the step-by-step synthesis, characterization and in vitro testing of FA-conjugated, RhB-labelled nanoparticles is discussed.

2.4.1 Preparation of fluorescent PGMA nanoparticles (RhB-PGMA Nps)

The chemical methodology adopted in this chapter presents the advantage of performing ligand conjugation post nanoparticle synthesis and the requirement of benign aqueous conditions for the same. Polymeric nanoparticles were synthesized using an o/w emulsification process. Before nanoparticle synthesis, the polymer was modified with RhB, using a previously reported procedure. The attachment occurs via an epoxide ring opening reaction between the freely available carboxyl group on RhB and epoxide group on PGMA. The modified polymer was dissolved in an organic phase containing methyl ethyl ketone (MEK) and chloroform. Then, as shown in figure 2.3, fluorescent nanoparticles were synthesized by emulsification of the organic phase into an aqueous phase containing Pluronic F108 as a surfactant, followed by homogenization of the resultant o/w emulsion.

Figure 2.3 Preparation of fluorescent PGMA nanoparticles. PGMA was reacted with RhB to render a fluorescent polymer which was used for the preparation of nanoparticles via an emulsification route (schematic representation).
2.4.2 Propargylation of RhB-PGMA Nps

To make the nanoparticle surface amenable to CuAAC ‘click’ reaction usually requires the application of an alternate coupling reaction and multiple purification steps (as mentioned in section 2.3.4). However, herein the versatile chemistry of the epoxide functionality helps avoid the extra preparatory steps. PGMA nanoparticles were conveniently functionalized with an alkyne group via an epoxy ring opening reaction between propargylamine and the epoxide groups of PGMA, conducted in aqueous conditions at room temperature. Unreacted propargylamine was easily washed off with water yielding propargylated fluorescent nanoparticles (Prop-RhB-PGMA Nps) (Figure 2.4).

Figure 2.4B, FTIR spectrum (b) for RhB labelled PGMA nanoparticles shows characteristic absorption bands for deformation of the epoxide ring at 908 cm\(^{-1}\) and 847 cm\(^{-1}\), which distinctly reduce in intensity upon propargylamine attachment in (a). Propargylamine, as shown in figure 2.4B(c) has the characteristic C-H bend (\(\text{C}==\text{C}==\text{H}\)) at 636 cm\(^{-1}\). A corresponding new band at 652 cm\(^{-1}\) appears in (a), confirming the introduction of terminal alkyne groups on the polymer. The presence of a primary amine stretch (\(\text{C}==\text{N}==\text{H}\)) in propargylamine shows two distinct bands at 3290 cm\(^{-1}\) and 3376 cm\(^{-1}\) that reduce to a single band for secondary amine stretch in (a). Comparison of the IR spectrum for Prop-RhB-PGMA Nps with that of RhB-PGMA Nps indicates disappearance of bands between 1600-1500 cm\(^{-1}\) which correspond to aromatic (\(\text{C}==\text{C}==\text{C}\)) stretch for RhB. This could result from the hydrolysis of the ester bonds under basic conditions. Importantly in the present case the nanoparticles still retained fluorescence for visualization under the microscope, indicating that not all the RhB was removed via ester hydrolysis. Furthermore, UV-vis analysis of the final product, FA-RhB-PGMA Nps following click reaction, as shown in figure 2.6C confirms a maxima at 564 nm, which characterizes the presence of RhB.\(^{338}\)
2.4.3 Synthesis of azide functionalized folic acid (azido-folic acid)

An azide functionality was introduced onto FA according to a previously reported method. This involved an NHS/DCC carbodiimide cross-linking reaction with formation of an NHS ester of FA, followed by the addition of 3-azido 1-propylamine as shown in figure 2.5A. The primary amine on 3-azido 1-propylamine coupled with the activated glutamate moiety on FA, to yield the desired azido-folic acid product. Analysis of the FTIR spectrum for azido-folic acid (Figure 2.5B(a)) shows a band at 2100 cm\(^{-1}\) corresponding to the characteristic peak for azide (\(\text{N} - \text{N} - \text{N} \)) functionality as observed in the reactant, 3-azido 1-propylamine (Figure 2.5B(c)). Figure 2.5C shows \(^1\)H NMR spectra for FA (bottom) and azido-folic acid (top) referenced to peak for d\(_6\)-DMSO (\(\delta = 2.50 \text{ ppm}\)).
Successful coupling of 3-azido 1-propylamine with FA was confirmed from NMR spectra for the product i.e. azido-folic acid (top) showing, $^1$H (400MHz, d$_6$-DMSO, 25°C) at $\delta$ (ppm): 3.45-3.39 (m, 2H, -CH$_2$N$_3$), 3.11-3.03 (m, 2H, -CONHCH$_2$CH$_2$N$_3$), 1.66-1.55 (m, 2H, -CONHCH$_2$CH$_2$N$_3$). These peaks are indicated by numbers 1, 3 and 2 in figure 2.5C (top), respectively. For FA (Figure 2.5C, bottom), the broad singlet at $\delta = 12.27$ ppm (s, 1H, -CONHCH$_2$CH$_2$COOH) reduced, indicating that FA was mostly functionalized into azido-folic acid via the $\gamma$-carboxylate of the glutamic acid moiety. Taken together, both FTIR and NMR results confirm the successful synthesis of azido-folic acid that was obtained at 72% yield.
Figure 2.5 Synthesis of azide functionalized FA (Azido-Folic acid). (A) Reaction scheme for synthesis of Azido-Folic acid. (B) FTIR spectrum for (a) Azido-folic acid synthesized by conjugation of (b) Folic acid with (c) 3-Azido 1-propylamine. (C) $^1$H NMR spectra for FA (bottom) and azido-folic acid (top) in d$_6$ DMSO.

2.4.4 “Click” conjugation for folate-RhB-PGMA nanoparticles synthesis

The availability of a terminal alkyne on the nanoparticle surface and the presence of an azide functionality on FA facilitated their coupling using a CuAAC click reaction. The reaction was performed under an atmosphere of argon in weak basic conditions, using ammonium bicarbonate buffer (pH 8.0, 10mM) at room temperature for 12 h (Figure
2.6A). These conditions are advantageous to maintain the biological activity of FA since it is sensitive to heat, light and variation in pH from neutrality. The copper-(I)-catalyst was generated in situ by reducing copper-(II)-sulfate with a freshly prepared solution of sodium L-ascorbate. Nanoparticles were extensively washed to remove unreacted azido-folic acid, and further dialyzed to remove trace amounts of copper. Successful conjugation of the folate moiety onto the nanoparticles was confirmed from FTIR spectra (figure 2.6B), wherein there is appearance of a new peak at 1607 cm\(^{-1}\) in (c) corresponding to (\(-\text{C}--\text{N}\)-) stretch of the 1, 2, 3 triazole ring\(^{341}\) formed as a result of the CuAAC cycloaddition. Furthermore, the peak corresponding to the azide functionality at 2100 cm\(^{-1}\) appearing in (a) was absent in (c) indicating absence of any reactant residue. However, the presence of a residual peak for free terminal alkyne groups (652 cm\(^{-1}\)) indicates an incomplete reaction that could be both due to early termination of the reaction or an incomplete reaction resulting from steric hindrance to azido-folic acid. FA attachment to the nanoparticles was also confirmed by UV-vis absorption spectra of Folate-RhB-PGMA-Nps (Figure 2.6C) which contained characteristic peaks for both FA and RhB at 358 nm and 564 nm, respectively.
Figure 2.6 Synthesis of FA-conjugated nanoparticles. (A) Schematic showing copper catalyzed “click” conjugation of azido-folic acid to propargylated RhB-PGMA nanoparticles to give FA-conjugated nanoparticles. (B) FTIR confirmation for click reaction showing (a) FA-RhB-PGMA Nps synthesized by CuAAC reaction between (b) Propargyl-RhB-PGMA Nps and (c) Azido-folic acid, and (C) UV-vis absorption spectrum for Folate-RhB-PGMA Nps confirming presence of FA and RhB on nanoparticles.

2.4.5 Nanoparticle characterization

Following the synthesis and purification of RhB-PGMA and FA-RhB-PGMA Nps, they were characterized for their physicochemical characteristics. The nanoparticle morphology was assessed using transmission electron microscopy (TEM) and dynamic light scattering (DLS) was used to determine their hydrodynamic diameter and surface potential.

As shown in figure 2.7A, the TEM image suggests a spherical morphology for FA-RhB-PGMA nanoparticles. Analysis of the average hydrodynamic diameter of nanoparticles (Figure 2.7B) revealed a shift from 193 nm for RhB-PGMA nanoparticles to 223 nm for FA-RhB-PGMA nanoparticles. This ~20 nm increase in average hydrodynamic
diameter following FA attachment to the nanoparticles could presumably result from the both the presence of an additional FA layer on nanoparticle and importantly from the resulting more solvated nanoparticle surface. Figure 2.7C shows change in zeta potential for the nanoparticles at each of the two steps leading to FA conjugation. There was a clear change in surface potential from near neutral to +29.4 mV on propargylation of the nanoparticles, which then reversed to a negative surface charge (-33.4 mV) on FA attachment.

Figure 2.7 Physicochemical characterization of Folate-RhB-PGMA-Nps. (A) A low magnification TEM image of FA-RhB-PGMA nanoparticles (scale bar – 250 nm), (B) Shows particle size before (open) and after (solid) FA attachment and (C) Shows the change in zeta potential with surface functionalization of nanoparticles.

Further, the amount of FA attached to nanoparticles was calculated using UV-vis spectrometry. Briefly, FA solutions of known concentration were prepared using ammonium bicarbonate buffer (10 mM, pH 8.0) and their maximum absorbance were observed at 358 nm to generate a standard curve for FA as shown in figure 2.8A. Then, the absorbance spectra of equivalent and known concentration of RhB-PGMA Nps and FA-RhB-PGMA Nps were observed. The difference in absorbance of RhB-PGMA and FA-RhB-PGMA nanoparticles at 358 nm as seen in figure 2.8B is attributed to the amount of FA present on the latter.
For a 0.23 mg/ml nanoparticle solution, the difference in absorbance was 0.65 a.u. (Figure 2.8B) which corresponds to 0.034 mg/ml of FA content from the standard curve. Calculating further, it results in 0.034 mg of FA per 0.23 mg of FA-RhB-PGMA nanoparticles, i.e. 14.6 mg/g or a 14.6 % (w/w) of FA content on FA-RhB-PGMA Nps.

![Standard curve](image)

Figure 2.8 FA content on nanoparticles, (A) Standard curve for FA generated using UV-vis absorption at 358 nm for FA solutions of known concentrations prepared using ammonium bicarbonate buffer (10mM, pH 8.0). Plot (B) is used to calculate amount of folate attached per unit weight of nanoparticles. Attributed by the amount of folate attached, 0.23 mg/ml of Folate-RhB-PGMA nanoparticles have an absorbance of 0.65 a.u. at 358 nm. This absorbance value is correlated with the corresponding FA concentration from the standard curve to calculate amount of FA attached to nanoparticles.

2.4.6 In vitro testing: FRα expression analysis and FA-RhB-PGMA Nps toxicity assessment.

Previous sections demonstrated the successful synthesis of actively targeting FA-RhB-PGMA nanoparticles and their physicochemical characterization including the confirmation and quantification of the targeting ligand attachment. Following this, the biocompatibility and innate toxicity of these nanoparticles was assessed. Briefly, three different cell lines; 1. HAL-15, Primary cells from human liver, 2. SKOV-3, ovarian cancer cells and, 3. A549, lung adenocarcinoma cells were incubated with RhB-PGMA and FA-RhB-PGMA Nps in cell culture media, at a concentration of 1 µg/ml, 10 µg/ml and 100 µg/ml for a period of 72 h each. Cell viability was tested using an MTS assay. As shown in figure 2.9 both RhB-PGMA and FA-RhB-PGMA Nps did not display any significant toxicity (ANOVA with Tukey’s post hoc, p>0.05, n=3) in any of the SKOV-3 (Figure 2.9A), HAL-15 (Figure 2.9B) and A549 cell lines (Figure 2.9C).
Figure 2.9 Nanoparticle toxicity assessment in vitro. Cell viability was tested post incubation with Folate-RhB-PGMA-Nps (FA-Nps) and RhB-PGMA-Nps (Nps) at a concentration of 1 µg/ml, 10 µg/ml and 100 µg/ml for 72 h and compared with untreated control (grey) for (A) SKOV-3 (ovarian cancer) cells, (B) HAL-15 (primary cells from normal human liver) and (C) A549 (lung adenocarcinoma) cells.

Before assessing the active targeting capability of FA-RhB-PGMA Nps for ovarian cancer cells, it is important to confirm the expression of FRα receptor on the chosen SKOV-3 cell line and determine its overexpression relative to other control cell lines. A flow cytometric analysis for FOLR1 receptor expression was performed on SKOV-3, A549, HAL-15 and HepG2 cells (liver adenocarcinoma cells) using an Allophycocyanin (APC) tagged human FOLR1 mAb (FRα antibody) and an APC tagged mouse IgG1 K Isotype control. As shown in figure 2.10, the receptor is highly overexpressed on SKOV-3 cells compared with the other cell lines.
Figure 2.10 Flow cytometric analysis and comparison of FRα (FOLR1) expression on SKOV-3 cells (using APC tagged anti-FOLR1 antibody) relative to normalized expression on A549 (lung-adenocarcinoma), HAL15 (primary cell line isolated from normal human liver) and HepG2 (Liver-hepatocellular carcinoma) cells.

2.4.7 Cellular uptake study

The targeting ability of FA-RhB-PGMA was confirmed in vitro using confocal laser scanning microscopy. Briefly, FRα overexpressing SKOV-3 cells and FRα deficient A549 cells were plated on glass coverslips placed in a 12 well plate (50,000 cells per well). After 24 h of incubation, cells were washed and cell culture media was replaced with fresh culture media containing either 10 µg/ml of RhB-PGMA Nps or 10 µg/ml of FA-RhB-PGMA Nps. After a period of 12 h of incubation with nanoparticles, cell samples were fixed and stained using a primary mouse α-tubulin mAb following up with a secondary Alexafluor-488 tagged goat anti-mouse IgG antibody. A hoechst solution was used for nuclear staining and samples were mounted onto glass slides for confocal microscopic analysis. A series of Z-stacks were taken for each of the samples and images presented in figure 2.11 are a maximum projection of the Z-stacks.

Figure 2.11A shows SKOV-3 (top, left and right) and A549 (bottom, left and right) incubated with non-targeted, RhB-PGMA-Nps. Images on the left show SKOV-3 (top) and A549 (bottom) cells stained green for (Alexafluor-488) α-tubulin. Images on right show cell nuclei stained blue and internalized RhB-PGMA Nps in red for both SKOV-3 (top) and A549 (bottom) cells. These cellular uptake images for FA-RhB-PGMA-Nps
suggest that both SKOV-3 and A549 cells were capable of internalizing a small amount of nanoparticles via a non-FR directed mechanism.

Similarly, figure 2.11B shows SKOV-3 (top, left and right) and A549 (bottom, left and right) incubated with FRα targeted, FA-RhB-PGMA-Nps. Images on the left show SKOV-3 (top) and A549 (bottom) cells stained green for (Alexafluor-488) α-tubulin. Images on right show cell nuclei stained blue and internalized FA-RhB-PGMA Nps in red for both SKOV-3 (top) and A549 (bottom) cells. The uptake of FA-RhB-PGMA Nps in SKOV-3 was clearly much higher than in A549 cells.

Studying figures 2.11A and 2.11B together, there is a clear positive correlation between the presence of FA on nanoparticles and their higher uptake in FRα overexpressing SKOV-3 cells. Furthermore, nanoparticle uptake in the A549 cells remains unaffected by FA conjugation. Taken together with the the flow cytometric results (Figure 2.10), this data confirms FRα receptor-mediated selective uptake of FA-RhB-PGMA nanoparticles in ovarian cancer cells.
Figure 2.11 Nanoparticle uptake in FRα overexpressing and FRα deficient cell lines. (A) RhB-PGMA-Nps in SKOV-3 (top) and A549 (bottom) cells, and (B) Folate-Rhb-PGMA-Nps uptake in SKOV-3 (top) and A549 (bottom) cells (Scale bar = 100 µm). Images on left from cells stained green (Alexaflour-488) for α-tubulin and right, cell nuclei stained blue (Hoechst) and nanoparticles in red (RhB).
2.5 Conclusion and future directions

Despite the clinical success of nanoparticle-based drug delivery systems in reducing chemotherapy related systemic effects, improved clinical outcomes in the form of significant enhancement in patient survival rate still remains unrealized. For this reason, there is a great deal of research interest focused on the development of multifunctional nanoparticles with active targeting capabilities. However, with the addition of every new functionality comes the additional ‘cost’ of synthetic steps, and financial and regulatory hurdles. A trade-off between the two is of utmost importance for successful bench to bedside translation of a nanoparticle formulation. It is also likely that targeted nanoparticles that minimize this cost to benefit ratio would perhaps be the earliest to be translated and utilized in the clinic. One of the important determinants of the cost associated with multifunctional nanoparticles is the conjugation technique to be used for their functionalization. The chemistry to be employed is dependent on the targeting ligand of interest, its required orientation and sensitivity to chemical environment as well as the material platform onto which it is to be attached.

In this chapter, successful and efficient conjugation of FA onto the PGMA nanoparticles via two easy steps demonstrates the promising potential of PGMA nanoparticles as facile platforms for bioconjugation. The click chemistry approach adopted here facilitates reduction in the number of chemical steps as well as the amount of ligand required. Furthermore, the possibility of conducting click reaction on PGMA nanoparticles in mild aqueous conditions affords the extension of its application for attachment of sensitive biomolecules as targeting ligands. The absence of undesirable byproduct further reduces the downstream costs associated with product purification.

As synthesised, both non-conjugated nanoparticles and actively targeted, FA conjugated nanoparticles (FA-RhB-PGMA Nps) were confirmed for their biocompatibility in vitro in primary cells from the human liver (HAL15), ovarian cancer cells (SKOV-3) and lung adenocarcinoma cells (A549). These nanoparticles did not effect cell viability up to a 100 µg/ml nanoparticle exposure over a period of 72 h. FA conjugated nanoparticles demonstrated much higher uptake in the FRα overexpressing SKOV-3 (ovarian cancer) cells in comparison with FRα deficient A549 (lung adenocarcinoma) cells, visualised using confocal laser microscopy. This difference in uptake was not evident for non-conjugated nanoparticles suggesting that increased cellular internalisation of FA attached nanoparticles is FRα-mediated.
The conjugation approach presented here is expected to demonstrate better efficiency than the traditionally used carbodiimide coupling reaction for folic acid conjugation onto nanoparticles. To date, there have been only a few studies comparing the two methodologies,\textsuperscript{342,343} and no direct quantitative comparison was made in this study. However, the versatility of the PGMA nanoparticle surface chemistry makes it a good platform to conduct a quantitative comparison between the two widely used conjugation methodologies. Secondly, considering the availability of a large number of epoxide functionalities on the PGMA nanoparticles, and the good efficiency of click conjugation chemistry, it may well be possible to readily control the density of conjugated ligand on the nanoparticles by altering the molar ratios of the reactants. Generation of a library of nanoparticles with specifiable surface ligand density can aid in studying the effect of ligand density on nanoparticle uptake and further optimization of the same. This is especially important for ligands such as FA, which have a concentration-dependent affinity for their receptors. Finally, although not addressed here, the additional advantage of nanoparticulate delivery system is their potential to carry multiple payloads. The ligand-tethered nanoparticles presented here can be further investigated for targeted theranostic applications by either loading or conjugating them with an anticancer agent.
2.6 Detailed methods

2.6.1 Nanoparticle synthesis and characterization

Materials

PGMA, poly(glycidyl methacrylate) (Mn = 220,515 g/mol, Mw = 433,730 g/mol polydispersity index = 1.97) was synthesized by Prof. Igor Luzinov and Dr. Bogdan Zydyrko, (School of Material Science and Engineering, Clemson University, Clemson, SC, USA), Rhodamine B (RhB, Kodak, 95%), Methyl ethyl ketone (MEK) (>99%, Fischer Chemical), Chloroform (CHCl₃) (>99%, Chem-Supply), Propargylamine (>99.8%, Sigma Aldrich), Pluronic F108 (Sigma Aldrich), 3-azido 1-propylamine (>99.7%, Alfa Aesar), DMSO (>99%, Sigma Aldrich), Folic Acid (>97%, Sigma Aldrich), Dicyclohexylcarbodiimide (DCC) (>98%, Sigma Aldrich)

Synthesis of fluorescent PGMA Nanoparticles (RhB-PGMA-Nps)

PGMA was synthesized by radical polymerization according to a published procedure. Briefly, glycidyl methacrylate was polymerized in MEK to produce PGMA (Mn = 220,515 g/mol, Mw = 433,730 g/mol, polydispersity index = 1.97), using azobisisobutyronitrile as initiator. The polymer was purified by multiple precipitations from MEK into diethyl ether. To attach dye to polymer, a solution of RhB (20 mg) and PGMA (100 mg) in MEK (50 ml) was heated at 70°C for 5 h. This solution was then reduced in vacuo and the resulting RhB-PGMA polymer was precipitated in diethyl ether and washed three times with ether to remove ungrafted RhB. The polymer was then dried and weighed. For nanoparticle synthesis, RhB-PGMA (100 mg) was dissolved in a 1:3 mixture of CHCl₃ and MEK (8 ml) to make up the organic phase. Nanoparticles were prepared by non-spontaneous emulsification of the organic phase in an aqueous solution of Pluronic F108 (1.25% w/v, 60 ml). The emulsion was homogenized with probe-type ultrasonicator for 1 min at low power. Organic solvents were allowed to evaporate under a slow flow of N₂ and stirring at 125 rpm overnight. The resulting solution was centrifuged at 3000g for 30 min to remove excess polymer which pellets out. The supernatant was collected and centrifuged at 20,000g for 30 min to collect the nanoparticles, which were resuspended in 10 ml of Pluronic solution.
(0.125% w/v) for storage till further use. Equivalent dry mass of nanoparticles was determined following freeze-drying of a known volume of nanoparticle dispersion.

**Synthesis of propargylated nanoparticles (Prop-RhB-PGMA-Nps)**

Nanoparticles were resuspended in milliQ water at a final concentration of 2 mg/ml. Propargylamine (90 µl, 2 equiv.) was added dropwise to 50 ml of the nanoparticle dispersion under stirring. The reaction mixture was left to stir under an atmosphere of argon for 24 h at room temperature. Unreacted propargylamine was removed by repeated washing and centrifugation (x4, 35 ml milliQ water each wash) at 20,000 g for 30 min. Equivalent dry mass of nanoparticles was determined after freeze-drying. Successful propargylamine attachment was confirmed using FTIR (Perkin Elmer, Spectrum one instrument with an ATR attachment).

**Preparation of azide functionalized FA (Azido-Folic acid)**

A terminal azide functionality was introduced onto FA according to a previously reported method. 500 mg of folic acid was dissolved in DMSO (20 ml) containing 250 µl of triethylamine. To this solution, 260 mg of NHS (2.2 equiv.) and 250 mg of DCC (1.1 equiv.) were added and stirred at room temperature over 24 h. Then, 0.24 g of 3-azido 1-propylamine (2 equiv.) was added into the reaction mixture which was stirred over another 24 h. The precipitated side product, dicyclohexylurea was removed by filtration and the crude product was precipitated in ethyl acetate. The crude product was dried overnight under vacuum, dissolved in 1 M NaOH, precipitated in 1 M HCl and purified by repeated washing and centrifugation (x4, ethanol/water 1:1, 30 ml each wash). The obtained final product was dried under vacuum, and its identity was confirmed by FTIR (Perkin Elmer, Spectrum one instrument with an ATR attachment) and NMR (1H, d6-DMSO, 399.868 MHz, Varian 400 WB spectrometer). The yield was 72%.

**Synthesis of FA conjugated nanoparticles using the “Click” chemistry approach**

Dispersions of propargylated nanoparticles (12 ml, 2 mg/ml) and azido-folic acid solutions (5 ml, 9 mg/ml) were separately prepared in freshly made NH₄HCO₃ buffer (10 mM, pH 8.0) and mixed together while stirring. A freshly prepared sodium L-ascorbate solution (50 mole% to the azido group, in NH₄HCO₃ buffer) followed by CuSO₄·5H₂O solution (20 mole% to the azido group, in NH₄HCO₃ buffer) were added,
and the mixture was left to stir at room temperature for 12 h under an atmosphere of argon. Afterwards, the reaction mixture was centrifuged at 20,000g for 30 min to collect the pelleted nanoparticles. Nanoparticles were repeatedly washed to remove unreacted azido-folic acid (x4; 30 ml NH₄HCO₃ buffer, 10 mM, pH 8.0, each wash), and dialyzed (Spectra/Por® Float-A-Lyzer® MWCO=100kDa) over 24 h against a solution of 0.125% (w/v) Pluronic F108 dissolved in 2 L of NH₄HCO₃ buffer (10 mM, pH 8.0). Successful attachment and presence of FA were confirmed using FTIR and UV-Vis absorption spectroscopy. The amount of FA present per unit mass of nanoparticles was calculated from a FA standard curve prepared previously.

Nanoparticle characterization

Nanoparticle samples were prepared at 0.20 mg/ml in Pluronic F108 (0.125% w/v in milliQ water) for size and zeta potential measurements. Both size and surface charge were measured in triplicate using the Zetasizer Nano series ZEN 3600 (Malvern Instruments). Each measurement was averaged over 12 runs for size and 100 runs for zeta potential. Samples for TEM were prepared by deposition and drying of 10 µl of the nanoparticle dispersion onto carbon-coated grids. TEM images were taken at 120 kV on a JEOL JEM-2100 microscope.

2.6.2 In-vitro testing

Cell culture materials

SKOV-3 cells (HTB-77™, passage 81) and A549 Cells (CCL-185™, passage 27) were purchased from the ATCC® (American Type Culture Collection, Manassas, VA 20108 USA). HAL-15, primary cell lines isolated from normal human liver and HepG2 (hepatocellular carcinoma cells, ATCC® HB-8065™, American Type Culture Collection, Manassas, VA 20108 USA) were a kind gift from Prof. George Yeoh, The University of Western Australia. All cells were maintained in folate free RPMI 1640 culture medium (GIBCO®) supplemented with 100 units/ml penicillin G, 100 µg/ml streptomycin (Sigma Aldrich) and 10% fetal bovine serum (FBS) (Invitrogen). MTS reagent ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] : CellTiter 96® AQueous One Solution (Promega) was used as an MTS reagent for cell proliferation assay. For Immunofluorescence: An α-tubulin (DM1A) mouse mAb (Cell signalling technologies®) was used as the primary antibody; AlexaFlour 488 Goat Anti-Mouse IgG antibody (Molecular Probes®) was used for
secondary staining. Antibodies for flow analysis of receptor expression: Allophycocyanin (APC) human FOLR1 mAb (FRα) antibody (R&D systems) and APC tagged mouse IgG1 K Isotype control (R&D systems). Antibody diluent: PBS (phosphate buffered saline) with 10% normal goat serum (NGS) (Invitrogen) and 0.1% Triton-X (Sigma Aldrich).

**Flow cytometric analysis of FOLR1 receptor expression**

Cells were harvested from an ongoing subculture flask (75 cm² surface area, canted neck, Greiner cell culture flask supplied by Sigma Aldrich, Australia) and counted to make two sets of 5x10⁵ cells in triplicate in 1.5 ml microcentrifuge tubes (Eppendorf®, supplied by Sigma Aldrich, Australia) The cell samples were washed with FACS Buffer (x1, PBS with 2% FBS, pH 7.4) and resuspended in 40 µl of the FACS buffer. 10 µl of Allophycocyanin (APC) tagged human FOLR1 mAb (FRα) antibody (for test samples) and 10 µl of APC tagged mouse IgG1K Isotype control (for control samples) were added and mixed into each of the cell samples in the two sets respectively. Samples were then incubated at 4°C for 30 min in the dark. Post incubation, the excess antibody was removed by washing (x2) with 500 µl FACS buffer followed by centrifugation at 300g for 5 min. Pelleted cells were resuspended in 200 µl FACS buffer and kept on ice until analysis. Analysis was done using BD LSRFortessa™ SORP cell analyzer (BD Biosciences) within 1 h of sample preparation. APC fluorescence was detected using a 660/20 band pass filter. A total of 80,000 events were recorded and further gated for single cells that were analyzed for FOLR1 expression. Data was analyzed using FlowJo Analysis software.

**Toxicity studies**

SKOV-3 (passage 83), A549 (passage 31) and HAL-15 cells were separately seeded in 12 well plates (Greiner Bio-One Cell star, supplied by Sigma Aldrich) at a density of 50,000 cells per well with 2 ml of folate free RPMI 1640 culture medium (GIBCO®) (supplemented with 100 units/ml penicillin G, 100 µg/ml streptomycin and 10% fetal bovine serum). After an overnight incubation at 37 °C in 5% CO₂ the cells were washed with pre-warmed Dulbecco’s Phosphate Buffered Saline (DPBS) and incubated with RPMI 1640 culture medium (GIBCO®) (supplemented with 100 units/ml penicillin G, 100 µg/ml streptomycin (Sigma Aldrich) and 10% FBS (Invitrogen)) containing RhB-PGMA Nps and folate-RhB-PGMA Nps at concentrations of 1 µg/ml, 10 µg/ml and 100
μg/ml. Control cells were incubated with culture media only. After 72 h of incubation at 37°C in an atmosphere of 5% CO₂, 200 μl of MTS reagent was added into each well and incubated for 3 h at 37 °C. Absorption was read at 492 nm using Enspire 2300 multimodal plate reader (Perkin Elmer). Experiments were conducted in triplicate, and one-way ANOVA with Tukey’s test at 95% confidence level was applied post hoc to compare unpaired means of absorbance values (n=3).

**Nanoparticle uptake studies**

SKOV-3 (passage 81) and A549 cells (passage 27) were plated at a density of 50,000 cells per well on poly-(L-lysine) coated glass coverslips placed in a 12 well plate. After 24 h of incubation in 2 ml of folate free RPMI 1640 culture medium (GIBCO®) (supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin and 10% FBS) cells were incubated for 12 h with either 10 μg/ml of RhB-PGMA Nps or 10 μg/ml of folate-RhB-PGMA-Nps prepared in culture media. After incubation, media and nanoparticles were removed and cells were washed (x3) with DPBS and fixed using paraformaldehyde solution (4% w/v in milliQ water). Fixed cells were washed (x3) with PBS and further incubated with α-tubulin (DM1A) mouse mAb (1:4000 in antibody diluent) for 30 min at room temperature. The unattached antibody was removed by washing (x2) with PBS. These cells were further incubated with AlexaFlour-488 goat anti-mouse IgG antibody (1:400 in antibody diluent) for 30 min at room temperature. Unattached antibody was removed by washing with PBS (x2), and cells were incubated with Hoechst solution (1:10,000 in PBS) for 5 min. Cells were finally washed with PBS (x2) and samples were mounted on to glass slides using fluoromont gold mounting media for analysis using a confocal microscope (Leica TCS SP2, Nikon A1Si). Confocal images presented in this chapter are maximum projections of Z-Stacks.

**Statistical analysis**

Statistical comparisons were made using one-way ANOVA (p<0.05), with Tukey’s (corrected for multiple comparison) post-hoc test. (GraphPad Version 6.0)
Chapter 3

Transferrin Receptor Targeted Multifunctional Nanoparticles: Evaluation of Efficacy in an Orthotopic Model of PC3 Prostate Cancer

3.1 Passive and Active targeting: The two mechanisms for nanoparticle-mediated tumor targeting in vivo

Targeted drug delivery using nanoparticles is a rapidly progressing field for the treatment of many diseases, in particular cancer. There have been significant advances in the field to demonstrate that nanoparticle-based formulations can reduce off-target toxicity and improve drug bioavailability, two important factors responsible for low therapeutic indices when using conventional therapy. Drug delivery approaches using nanoparticles for the treatment of cancer have thus far been achieved on the basis of either passive or active targeting mechanisms. Both of these approaches have been reported to preferentially enhance the intracellular concentration of drugs in tumors while limiting their systemic side effects. The EPR effect in tumors constitutes an important mechanism for selective accumulation of nanoparticles in the tumor interstitium resulting in what is defined as passive targeting. In this case, high nutrient demanding tumor tissue is associated with rapid recruitment of new vessels (neovascularization) or rerouting of existing vessels near the tumor mass resulting from an imbalance of angiogenic regulators. This consequently leads to a highly disorganized and dilated tumor microenvironment with enlarged gap junctions between endothelial cells along with a compromised lymphatic drainage. This unique pathophysiology of tumor vessels has successfully been adopted to enable selective accumulation of nanoparticles in tumor tissues. On the other hand, active targeting is achieved via the attachment of ligands (small molecules, peptides, proteins) to the nanoparticle surface. This strategy enables nanoparticle recognition and binding to appropriate receptors that are overexpressed by tumor cells or tumor vasculature with
limited expression on normal cells. Receptor binding is then followed by nanoparticle internalization, thereby increasing specific uptake and reducing off-target toxicity encountered with passive targeting. However, the process of nanoparticle-mediated active targeting of tumor cells is preceded by their passive accumulation in tumors and this raises the issue, can an actively targeted drug formulation be entirely distinguished from a passive one. Furthermore, it is now widely accepted that interaction of nanoparticles with plasma proteins over time leads to the formation of a protein corona, resulting in screening of the targeting ligands on the surface of nanoparticles and a concomitant loss of active targeting capabilities.

In this study, the nanoparticle formulation dosage in vivo was accounted as an important variable to assess therapeutic distinction between the two processes. This was demonstrated by adopting a systematic dose dependent comparison between clinically used docetaxel formulation (Dtxl), non-functionalized, docetaxel loaded poly(glycidyl methacrylate) (PGMA) nanoparticle formulation for passive targeting (Dtxl-Np) and transferrin tethered, docetaxel loaded PGMA nanoparticle formulation (Tf-Dtxl-Np) for active targeting. These formulations were evaluated in a highly relevant orthotopic model (PC3) of castration-resistant prostate cancer (CRPC).

### 3.2 Castration-resistant prostate cancer and current treatment modalities

Prostate cancer is the second leading cause of cancer related death in men and the risk of mortality is higher in patients diagnosed with cancers of an aggressive and androgen insensitive subtype. In its initial stages, prostate cancer is primarily regulated by hormones, making androgen deprivation therapy (ADT) an effective therapeutic intervention. Importantly, following initial diagnosis and therapy, androgen-independent tumor cells are associated with clinical relapse leading to a malignant state termed the castration-resistant prostate cancer (CRPC). This state of castration resistance is associated with metastasis, poor prognosis and significant debilitation leading to depreciation of median patient survival to less than two years. A 75 mg/m² dose of docetaxel (Dtxl) based chemotherapy, once every 3 weeks in combination with oral prednisone is the first line treatment for CRPC. This schedule follows the conventional chemotherapy regimens based on administration of short bursts of antineoplastic drugs at high dose (at or near maximum tolerable dose; MTD), followed by periods for recovery of normal tissues, to balance off-target toxicity with therapeutic efficacy.
Unfortunately, this treatment regimen is associated with only modest survival rates but high incidence of systemic effects such as myelosuppression, neutropenia and hypersensitivity reactions. The intrinsic overexpression of prostate specific membrane antigen (PSMA) in prostate cancer cells has been widely exploited for development of targeted nanoparticle based therapeutics to overcome the aforementioned problems of the conventional therapy. However, in the case of CRPC cell lines, such as PC3 and DU145, overexpression of PSMA is not evident. Consequently, the inability to target PSMA in the case of CRPC results in disease progression attributed to their higher invasiveness and aggressive phenotype when compared to the PSMA expressing cells.

3.3 Transferrin receptor is overexpressed in CRPC and protein transferrin is a pertinent ligand for active targeting of PC3 prostate cancer

The activation of oncogenic signaling pathways regulated by c-myc and phosphoinositide 3-kinase (PI3K) are suggested to play a key role in the development of castration-resistance in prostate cancer. Importantly, the overexpression of the c-myc oncogene in prostate cancer is associated with elevated levels of transferrin receptor (TfR) mRNA. Additionally, tumors with activated PI3K pathway have upregulated HIF-1α transcription factor for which TfR is a direct target gene. The activation of these two pathways in CRPC are indicative of transferrin receptor overexpression, making it an optimal target for selective therapy of highly metastatic, castration-resistant PC3 prostate cancer.

TfR overexpression has been successfully exploited to develop targeted nanoparticle based therapeutics, including SGT-53, SGT-94, MBP-426 and CALAA-01 which are currently in their phase I/II of clinical trials. The latter two examples use protein holo-transferrin (hTf) as a targeting ligand, enabling receptor mediated endocytosis of nanoparticles and intracellular drug release. In the present case this indeed justifies the use of Tf as a ligand for evaluating active targeting by nanoparticles.
3.4 Multifunctional PGMA nanoparticles for active and passive targeting of PC3 prostate cancer

3.4.1 Preparation of multifunctional PGMA nanoparticles

The nanoparticles used in this study consisted of magnetite (Fe$_3$O$_4$) nanoparticles and drug Dtxl encapsulated within a Rhodamine B-poly(glycidal methacrylate) (RhB-PGMA) matrix. The epoxide functionality of PGMA was exploited to covalently attach RhB and transferrin to the nanoparticle surface by means of a simple ring-opening reaction (Figure 3.1A). The attachment of RhB and the encapsulation of magnetite nanoparticles enables the utilization of a multimodal approach to assess the efficacy of non-functionalized PGMA nanoparticle formulation for passive targeting (Dtxl-Np) and transferrin tethered PGMA nanoparticle formulation (Tf-Dtxl-Np) for active targeting. In the present case ex vivo magnetic resonance relaxometry and in vitro fluorescence microscopy were used to evaluate biodistribution and targeting respectively. The presence of encapsulated magnetite in the formulations also enabled accurate means to separate, wash, and concentrate the nanoparticles using a magnetic fractionation column following surface functionalization. Magnetite nanoparticles (Figure 3.1B) were prepared by thermal decomposition of iron (III) acetylacetonate, using a method for synthesis of 6 nm magnetite nanoparticle as described by Sun et al.$^{378}$ Superparamagnetic behavior of these nanoparticles was determined using superconducting quantum interference device (SQuID) magnetometry (Appendix B). The polymeric nanoparticles were synthesized using a nonspontaneous emulsification method, in which a binary solvent mixture containing both immiscible and soluble components were employed as the dispersed phase. This organic solution of the RhB-modified polymer, also containing magnetite nanoparticles and Dtxl, was emulsified in water in the presence of a surfactant (Pluronic F108). The purified nanoparticles were characterized using TEM (Figure 3.1C, D) and DLS (Figure 3.1E). Figure 3.1C, D show polymeric nanoparticles (larger spheres) encapsulating higher density (darker; smaller spheres) magnetite nanoparticles. Drug loading and drug release kinetics were measured using reverse-phase high performance liquid chromatography (RP-HPLC) and protein attachment was measured using UV absorption at 280 nm.
Figure 3.1 Preparation and physical characterization of transferrin conjugated PGMA multifunctional nanoparticles. Fluorescent, superparamagnetic, drug loaded multifunctional nanoparticles were prepared by o/w emulsion method and h-Tf was tethered using reactive epoxide functionality of PGMA. (A) Schematic representation of Tf attachment to PGMA-RhB nanospheres (RhB-labelled; represented by pink) containing docetaxel and iron-oxide nanoparticles (magnetite nanoparticles; represented by imbeded grey smaller spheres). (B) TEM image of magnetite (Fe₃O₄) nanoparticles prepared by organic solution-phase decomposition (scale bar – 50 nm). (C) Low-magnification TEM image of polymeric nanoparticle (scale bar – 50 nm). (D) Individual polymeric nanoparticle showing distribution of magnetite nanoparticles within the polymer shell (scale bar – 200 nm). (E) Particle hydrodynamic size and (F) zeta potential distribution of nanoparticles before (open) and after (solid) h-Tfn attachment.
3.4.2 Physicochemical characteristics of multifunctional PGMA nanoparticles

Analysis of the hydrodynamic diameters of nanoparticles revealed a shift in average size from 156 nm (95% confidence interval 85.4 – 335.4 nm) to 183 nm (95% confidence interval 98.9 – 388.5 nm) following the attachment of transferrin to the nanoparticles (Figure 3.1E). This increase in the diameter is attributed to both the attachment of transferrin on the nanoparticles along with the resultant more solvated nanoparticle surface. Furthermore, transferrin attachment resulted in a noticeable shift in the zeta potential from 0.89 mV to -18.9 mV (Figure 3.1F). Protein holo-transferrin (diferric) has a pI of 5.6. Thereby, its presence on nanoparticle surface lead to a concomitant negative zeta potential (in PBS; pH 7.4). UV-vis spectrometric analysis further established a 3.7 ± 0.5% protein (w/w) attachment to the nanoparticles. The drug loading was measured to be 8.9 ± 0.5 % (w/w). In vitro drug release study demonstrated a cumulative release of 33.2 ± 1.2 % and 5.7 ± 0.4 % of the total drug load over a period of 120 h in the presence or absence of a biorelevant release medium respectively (which provided for improved drug solubility and sink conditions for release study) (Figure 3.2A). Characterization of magnetic properties of the polymeric nanoparticles using SQuID magnetometry confirmed their superparamagnetic characteristic with the zero field cooled/field cooled curve showing a maximum blocking temperature ($T_B$) of 65K (Figure 3.2C) and absence of magnetic hysteresis at 300K with a specific saturation magnetization of 8.4 emu/g (Figure 3.2D). The proton transverse relaxivity ($r_2$) of the nanoparticles was 297 s$^{-1}$ mM$^{-1}$ [Fe], based on the iron content inside the polymeric nanoparticles (Figure 3.2B). This value is higher than the proton transverse relaxivity ($r_2$) values for most clinically approved superparamagnetic iron oxide contrast agents such as Endorem/Feridex (120 s$^{-1}$ mM$^{-1}$), Resovist (189 s$^{-1}$ mM$^{-1}$), and Combidex/Sinerem (65 s$^{-1}$ mM$^{-1}$).380 Nanoparticles had 12.62 ± 0.53 % of iron (w/w), determined using ICP-AES analysis.
Figure 3.2 Drug release kinetics and magnetic characterization of transferrin conjugated PGMA multifunctional nanoparticles. (A) Cumulative docetaxel release from Tf-Dtxl-Nps in PBS (pH 7.4) and release medium (PBS + 0.1% w/w Tween 80) at pH 7.4 as sink conditions over a time period of 120 h. Relaxivity ($r_2$) is calculated from linear regression of plot (B) which shows transverse relaxation rate ($R_2$) versus iron concentration $[\text{Fe}]$ for Tf-Dtxl-Nps. (C) Shows zero field cooled (grey) and field cooled (black) curves for magnetite encapsulating polymeric nanoparticles ($T_B = 65K$). (D) Shows hysteresis loop for magnetite encapsulating polymeric nanoparticles at 5K (black) and absence of hysteresis at 300K (grey).

3.5 Establishment of active targeting in vitro using Tf-Dtxl-Nps.

PC3 cells were confirmed for their higher surface expression of the transferrin receptor (CD 71) relative to A549 (lung adenocarcinoma), HAL15, HAL23 (primary cell lines isolated from normal human liver) and HepG2 (liver-hepatocellular carcinoma) cell lines (Figure 3.3A) prior to assessment of the active targeting capability of Tf-Dtxl-Nps in vitro. Initially, the targeting capability of Tf-Dtxl-Nps was assessed by monitoring the increase in RhB fluorescence from the internalized nanoparticles using confocal live-cell imaging. Briefly, PC3 cells were incubated in cell culture media containing 10 µg/ml of Tf-Dtxl-Nps for 18.5 h and confocal imaging frames were taken at regular time intervals in this duration. Confocal imaging frames taken at times 0, 0.5, 4, 8, and 18 h are as shown in figure 3.3B. Mean fluorescence intensity per cell area from each of
the time-lapse imaging frame was calculated and plotted against time as shown in figure 3.3C. Having established highly effective uptake of nanoparticles with time, next the cytotoxicity of Tf-Dtxl-Nps was evaluated in PC3 cells and compared with equivalent Dtxl-Nps and free Dtxl. Cells were pre-incubated with treatment formulations over a period of 16 h, washed and further incubated in cell culture media for 72 h prior to conducting an MTS assay. The analysis revealed that active targeting using Tf-Dtxl-Nps results in significantly higher efficacy in comparison to the passively targeted Dtxl-Nps at 75 nM and 100 nM and significantly higher efficacy at all concentrations ranging from 1 nM to 100 nM in comparison with the clinical drug formulation (Figure 3.3D). It is noteworthy that nanoformulations without Dtxl were also tested as controls and showed no significant change in cell viability.
Figure 3.3 Analysis of transferrin receptor (CD-71) expression on PC3 cells, in vitro internalization of Tf-Dtxl-Nps in PC3 cells and their cytotoxicity assessment. Figure (A) shows flow cytometric analysis and comparison of transferrin receptor (CD-71) expression on PC3 cells relative to normalized expression on A549, HAL15, HAL23 and HepG2 cells. (B) Shows frames from live cell confocal time lapse imaging (0, 0.5, 4, 8, 18 h) showing internalization of multifunctional nanoparticles by PC3 cells (red = RhB, nanoparticles; scale bar = 100 µm). (C) Nanoparticle uptake in PC3 cells over time quantified by mean RhB fluorescence per cell area. (D) Cell inhibition determined at 72 h after an overnight exposure to Dtxl, Dtxl-Nps, Tf-Dtxl-Nps at 1.25, 7.50, 12.5, 75 and 125 nM concentration of docetaxel respectively. (*, data points for Tf-Dtxl-Nps statistically significant compared with both Dtxl and Dtxl-Nps, p < 0.5)

3.6 In vivo efficacy evaluation: Dosage is an important contributor to attribute active targeting in vivo.

Luciferase expressing PC3-LN cells were used in the in vivo orthotopic prostate cancer murine model to enable bioluminescence imaging (BLI) and a non-invasive analysis of tumor growth. Herein, PC3-LN cells were orthotopically implanted into the prostate of 7 to 8 week old male nude mice. These animals were then analyzed for tumor development over a period of 22 days followed by randomization into (i) NaCl saline control (n=8), and three test groups (n=10 each): (ii) clinical Dtxl formulation, (iii)
Dtxl-Nps and (iv) Tf-Dtxl-Nps at equivalent docetaxel dosage of 2 mg/kg, 5 mg/kg and 12 mg/kg. The highest concentration of docetaxel administered in this study, 12 mg/kg in mice, was chosen for its clinically trialed human equivalent dose (HED) of 36 mg/m². The treatment was administered via tail vein injections, once each day at days 24, 28 and 32, over the 37 day study period. Change in the luciferase activity over time was used to measure the efficacy of the formulations for tumor inhibition. Following the toxicity assessment as described by Farokhzad et al. and Li et al., animal weights were monitored every alternate day until the end of treatment as an indicator of the systemic toxicity associated with each treatment.143

It is evident that the treatment in the case of the clinical Dtxl formulation was effective at high drug doses (5 mg/kg and 12 mg/kg) (Figure 3.4A-C). However, these treatments resulted in concomitant significant weight loss in animals (Figure 3.4E, F), which is an indicator of the systemic off-target toxicity. While, the untreated (NaCl) animals gained a mean 7.0 ± 2.5% in weight, 6 of the 10 animals treated with 5 mg/kg Dtxl and 9 of the 10 animals treated with 12 mg/kg Dtxl lost weight, with a significant mean loss of 3.8 ± 2.5 % and 9.5 ± 2.7 %, respectively (Figure 3.4D-F). In contrast, there was no significant weight loss associated with Tf-Dtxl-Nps at any of the doses administered, and weight loss was evident only with the highest dose of 12 mg/kg (0.8 ± 2.2%) for the passively targeted Dtxl-Nps formulation. Importantly, in the context of active and passive targeting using nanoparticles, the Tf-Dtxl-Nps treatment was highly effective at the lowest dose of 2 mg/kg when compared to the passive Dtxl-Nps treatment (Figure 3.4A). In comparison with the untreated animals, the tumor growth for animals treated with 2 mg/kg Dtxl, 2 mg/kg Dtxl-Nps, and 2 mg/kg Tf-Dtxl-Nps was inhibited by a mean 15.7%, 62.6% and 94.6%, respectively. However, in animals administered with higher doses, there was no distinction in the efficacy of tumor control between the active and passive targeting formulations, suggesting that at these higher nanoparticle concentrations, the EPR effect in tumors constitutes an important mechanism that becomes the dominant determinant of the therapeutic outcome for both the active and passive targeting formulations.
Figure 3.4 Evaluation of *in vivo* efficacy of Dtxl, Dtxl-Nps, and Tf-Dtxl-Nps at three different drug doses. *In vivo* BLI was performed on day 8, 15, 22, 29 and 35 and intravenous injection treatment was administered on day 24, 28 and 32 (represented with ↓). (A-C) Change in luciferase activity in tumors for animals treated with (A) 2 mg/kg, (B) 5 mg/kg and (C) 12 mg/kg dose by docetaxel concentration. Change in luciferase activity for the saline treated group (NaCl; blue) indicates ~2.16 fold increase in tumor load over the 12 day treatment period. Significance was tested against untreated animals at day 22, 29 and 35. (*, p<0.05; **, p<0.01). (D-F) show changes in body weight (%) from day 23 (pretreatment weight) until day 37 (pre-necropsy weight) for animals treated with (D) 2mg/kg, (E) 5mg/kg and (F) 12mg/kg dose by docetaxel concentration. Animal weights were monitored on alternate days in duration of the study period. Statistical significance was tested against the untreated group and between formulation types for each dose ((*, p<0.05; **, p< 0.01; ****, p<0.0001). All data in (A-F) are presented as mean ± SEM. (G) shows representative mice BLI images at (a) Commencement (day 22) and (b) Treatment termination (day 35); bar shows color legend for BLI photon intensity.
3.7 Relaxometry results suggest hepatic clearance of nanoparticles

Finally, in vivo reticuloendothelial clearance of passive (Dtxl-Nps) and active (Tf-Dtxl-Nps) formulations was evaluated at the three concentrations post-necropsy using magnetic resonance relaxometry of the liver, spleen and kidney (Figure 3.5). The presence of magnetite in nanoparticles facilitated the use of rate of decay of spin echo recoverable proton transverse magnetisation (R2) within tissues as a surrogate indicator of nanoparticle accumulation (Y axis, Figure 3.5A-C). For high relaxivity superparamagnetic iron oxide (SPIO) based MRI contrast agents, such as nanoparticles used in this study \( r_2 = 297 \text{s}^{-1} \text{mM}^{-1} \), the tissue R2 values are a more sensitive measure for determining nanoparticle accumulation then the total tissue iron concentration. This means that the R2 for a tissue could change significantly for a very small, insignificant change in total iron concentration upon SPIO accumulation in a tissue. However, it is important to take into account both the total changes in iron concentrations in tissue and iron oxide nanoparticle induced changes in R2 in the assessment. We determined the total tissue iron concentrations [Fe], using ICP-AES analysis of lyophilized tissues to determine if there was any variability in tissue iron concentration with treatment (Figure 3.5D-F). This is important as it is widely accepted that iron deficiency (ID) and anemia are frequent complications in cancer patients, in particular during treatment with chemotherapeutic agents, which can cause natural variability in endogenous tissue iron concentrations with treatment.\(^{[21]}\) In the case of tumor tissue, analyzing the changes in R2 and total iron is further complicated and the sensitivity of R2 for indication of nanoparticle accumulation in the tumor tissue was insufficient (Appendix D). These complications result from first, the natural variation between tumors i.e. the tumor tissues established in different animals are heterogeneous and naturally variable animal to animal (especially noting that we established an orthotopic tumor). For this reason, these tumors may display very diverse initial endogenous tissue iron concentrations. Secondly, both the tumor vasculature and its microenvironment would change with time and with different forms of treatment influencing its iron concentration and R2 values. Herein, R2 values for Dtxl-Nps and Tf-Dtxl-Nps treated animals were compared with Dtxl and NaCl treated animals (Figure 3.5A-C). In the liver (Figure 3.5A), both nanoparticle formulations showed a trend towards higher R2 values relative to untreated and Dtxl treated animals at all concentrations. These differences in R2 were most apparent and reached statistical significance in the 12 mg/kg animals. Iron concentrations (Figure 3.5D) did not show a similar trend and there were no significant differences indicating the aforementioned better sensitivity of relaxometry to
nanoparticle accumulation. In the spleen (Figure 3.5B), significant accumulation of passive (Dtx1-Nps) nanoparticles was detected at the highest dosage and there were no significant differences in iron concentrations (Figure 3.5E). In the kidneys (Figure 3.5C), where large nanoparticles would not necessarily be expected to accumulate, relaxometry indicated some minor accumulation at 12 mg/kg for the passive nanoparticle formulation.
Figure 3.5 Transverse relaxation rate (R2) and iron concentrations in liver, spleen and kidney.

Transverse relaxation rate R2 for (A) liver, (B) spleen and (C) kidney and corresponding iron concentration, (D), (E) and (F) in the respective organs obtained using ICP-AES analysis of lyophilized organs. Data is reported as mean ± SD. * represents statistical significance relative to untreated group (blue bar); $, Y represent statistical significance relative to Dtxl (red bar) administered at 2 mg/kg, 5 mg/kg and 12 mg/kg, respectively. Other comparisons between groups as marked (p<0.05; significance by ANOVA with Tukey’s post hoc test for multiple comparisons; *, $, Y, p<0.05).
3.8 Conclusion

Successful cancer therapy is defined by an improvement in the quality of life and survival rate of patients. However, in the case of chemotherapy the frequency of such success is hindered by the systemic side effects during treatment. Results from this study in a PC3 prostate cancer orthotopic model are in confirmation that the clinically administered docetaxel formulation is potent only when administered at high doses, and this effectivity in tumor is accompanied with a dose dependent, significant loss in total body weight. Nanoparticle-mediated chemotherapy is better tolerated in animals compared with the clinical formulation as indicated by the change in animal weights over the study period. These observations together with the relaxometery study results that suggested hepatic clearance of nanoparticles, draws our attention to the fact that nanoparticles that miss their target organ are readily cleared by the liver before the chemotherapeutic can adversely affect healthy tissues. It is for this reason that nanoparticle mediated drug delivery provides an effective means for tumor targeting based on either a passive or an active mechanism.

The study further demonstrated that the difference in efficacy of the three formulations, Dtxl, Dtxl-Nps and Tf-Dtxl-Nps is dose dependent. While all the three formulations were significantly efficacious on the tumor load when administered at higher doses, the therapeutic distinction is evident at the lowest dose used (2 mg/kg; HED of 6 mg/m²), wherein facilitated by active transferrin receptor targeting, Tf-Dtxl-Nps demonstrated statistically significant tumor growth inhibition. This suggests that the utilization of transferrin tethered PGMA based multifunctional nanoparticle formulation is a potentially promising, systemically tolerable alternative that could allow reduction in clinically administered dose of docetaxel required for significant efficacy in CRPC.

Furthermore, this study also establishes that nanoparticle dosage is an important parameter that determines where the therapeutic efficacy of actively and passively targeted formulations becomes indistinguishable. The findings suggest that dosage needs to be accounted for when proposing the mode of action of the nanoparticles, hence providing a key fundamental outcome that will prove valuable in our understanding of the role of passive targeting in making an actively targeted formulation effective.
3.9 Materials and detailed methods

Materials

PGMA, poly(glycidyl methacrylate) (Mn = 220515 g/mol, Mw = 433730 g/mol polydispersity index = 1.97) was synthesized by Prof. Igor Luzinov and Dr. Bogdan Zydyrko, (School of Material Science and Engineering, Clemson University, Clemson, SC, USA). All chemicals were purchased from Sigma-Aldrich (Australia) unless otherwise stated: benzyl ether (99%), methyl ethyl ketone (MEK) (>99%, Fischer Chemical), chloroform (>99%, Chem-Supply), iron (III) acetylacetonate (97%), oleic acid (BDH, 92%), oleyl amine (70%), Pluronic® F108, rhodamine B (RhB; Kodak, 95%), human holo-transferrin (98%), docetaxel (97%, LC laboratories, Woburn, MA, USA), and 1, 2-tetradecandiol (90%) were used as received. All materials for cell culture were purchased from Invitrogen unless otherwise stated: DMEM, F12K medium, fetal bovine serum (FBS), normal goat serum (NGS), penicillin/streptomycin, trypsin/EDTA, PBS and DPBS. Allophycocyanin (APC) tagged anti-human CD71 antibody and APC tagged mouse IgG1 K isotype control were purchased from Affymetrix eBioscience.

3.9.1 Nanoparticle synthesis

Magnetite nanoparticles synthesis

Magnetite was synthesized by thermal decomposition of iron (III) acetylacetonate in benzyl ether at 300˚C in presence of oleic acid, oleyl amine and 1, 2 tetradecandiol in accordance with the method described by Sun et al.378 Magnetite nanoparticles were stored as a suspension in hexane and resuspended in chloroform before use.

Synthesis of RhB functionalized PGMA

PGMA was synthesized by radical polymerization according to a published procedure.338 Briefly, glycidyl methacrylate was polymerized in MEK to produce PGMA (Mn = 220,515 g/mol, Mw = 433,730 g/mol, polydispersity index = 1.97), using azobisisobutyronitrile as initiator. The polymer was purified by multiple precipitations from MEK into diethyl ether. To attach dye to the polymer, a solution of RhB (20 mg) and PGMA (100 mg) in MEK (50 ml) was heated to 70˚C for 5 h.338 The solution was then reduced in vacuo and the polymer was precipitated in diethyl ether and washed
three times with diethyl ether to remove ungrafted RhB. Polymer was then dried, weighed and redissolved in chloroform for nanoparticle synthesis.

**Polymeric nanoparticle synthesis**

Nanoparticles were prepared by modification of a non-spontaneous emulsification route as described by Evans et al.\textsuperscript{289} The organic phase contained magnetite nanoparticles (~24 mg), docetaxel (~24 mg) and RhB-PGMA (100 mg) in a 1:3 mixture of CHCl\textsubscript{3} and MEK (8 ml). The organic phase was added dropwise to rapidly stirring aqueous solution of Pluronic\textsuperscript{®} F108 (0.50% w/v, 2 x 30 ml). The emulsion was homogenized with a probe-type ultrasonicator for 1 min at low power. Organic solvents were allowed to evaporate under a slow flow of nitrogen and moderate stirring overnight. The resulting solution was centrifuged at 3000g for 30 min to remove excess polymer and magnetite aggregates and the supernatant was centrifuged at 20,000g for 30 minutes to collect nanoparticles. The magnetic polymeric nanoparticles were collected on a magnetic separation column (LS, Miltenyi Biotec), washed with Pluronic\textsuperscript{®} F108 solution (0.125% w/v, 2 x 1.5 ml), and then flushed with water until the filtrate ran clear. The resulting concentrated particle suspension was stored at 4°C for analysis and subsequent use. Equivalent dry mass of nanoparticle was determined by lyophilization.

Six batches of drug loaded nanoparticles synthesized separately were pooled together and then divided into two parts. One half was used as Dtxl-Np formulation and the other was used for transferrin attachment.

**Transferrin attachment to nanoparticles**

Transferrin was attached to nanoparticles by modification of a method previously described by Sahoo and Labhasetwar.\textsuperscript{384} The modification involved replacement of borate buffer, pH 5.0 (used by authors in the referenced paper) with acetate buffer, pH 5.0, for its better buffering capacity at this acidic pH. Acidic conditions were used because they are favourable for epoxide ring opening. The nanoparticle solution as prepared before was centrifuged at 20,000g for 30 min. Nanoparticles were collected and resuspended at 4 mg/ml in acetate buffer (10 ml, pH 5.0). To this, 5 ml of Tf solution in acetate buffer (8 mg/ml, pH 5.0) was added and stirred for 4 h at 37 °C. Unreacted Tf was removed by washing and centrifugation thrice (15 ml acetate buffer each wash). Nanoparticles were resuspended in Pluronic\textsuperscript{®} F108 solution (0.125% w/v)
and stored at 4 °C till further use. Equivalent dry mass of nanoparticles was determined by lyophilization.

### 3.9.2 Nanoparticle characterization

**Transmission Electron Microscopy**

Polymer nanoparticle samples for TEM were prepared by repeated washing with water and centrifugation to get a very dilute nanoparticle solution in water. Magnetite nanoparticle samples were prepared by diluting in hexane. 20 µl of each sample was deposited and air dried on carbon-coated copper grid. Images were taken on JEOL JEM-2100 microscope at 120 kV.

**Dynamic light scattering and Zeta potential measurement**

Nanoparticle samples were prepared at 0.20 mg/ml in Pluronic® F108 (0.125% w/v) for hydrodynamic size and zeta potential measurement. Both size and surface charge were measured in triplicate. Each measurement was averaged over 12 runs for hydrodynamic size and 100 runs for zeta potential.

**SQuID magnetometry**

Magnetic characterization was carried out using a Quantum Design 7 Tesla MPMS3 SQuID susceptometer. The zero field cooled and field cooled curves were collected in a 100 Oe field. The sample was cooled in zero field for the zero field cooled curve and was saturated in 7T at the maximum temperature prior to cooling in the measurement field for the field cooled curve. Hysteresis loops in fields up to 7 T were also measured at 300 K and at 5 K.

**High Performance Liquid Chromatography**

**Drug loading determination**

Lyophilized nanoparticles were suspended in methanol at a concentration of 1 mg/ml. Samples prepared in triplicate were sonicated using a water bath for ~10 sec once every 15 min for 1 h to dissolve the entrapped drug. Nanoparticles were centrifuged (14,000g, 30 min) into a pellet and the supernatant was collected for analysis. 120 µl aliquots of supernatant from each of the three samples after drug extraction were analyzed on a Waters 2695 HPLC instrument with a Waters 2489 UV/Vis detector using reverse phase
isocratic elution through a C18 column (150 x 4.60 mm, 5 µm, 25 ± 5°C). A detection wavelength of 232 nm was used for docetaxel. The mobile phase constituted methanol: water (70:30; v/v) at a flow rate of 1.5 ml/min over a run time of 12 min. An injection volume of 20 µl was used for each sample in triplicate. The area under the curve at retention time ~ 8 min was integrated and drug concentration was calculated from a standard curve (R²=0.9999) prepared using equivalent run conditions (Appendix A). Drug loading was calculated as,

\[
\text{Drug loading (\%)} = \frac{\text{mass}(\text{docetaxel in nanoparticles})}{\text{mass}(\text{nanoparticles})} \times 100
\]

All values are quoted as mean ± SD.

**In vitro drug release studies**

*In vitro* drug release was measured by dialysis in triplicate. In brief, 10 mg of docetaxel loaded nanoparticles were dispersed in either 5 ml of PBS (pH 7.4) or 5 ml of release medium (PBS, pH 7.4 with 0.1% w/v Tween 80). These suspensions were put in 5 ml volume dialysis tubing (Spectra/Por® Float-A-Lyzer® MWCO=100 kDa). The dialysis tubes were immersed in a centrifuge tube containing either 15 ml PBS or 15 ml release medium, respectively. The tubes were then placed in an orbital shaker at 125 rpm, 37°C. At each time interval, 10 ml of samples were removed and replaced with fresh buffer/medium to maintain flux conditions. Samples for analysis were lyophilized and docetaxel was extracted from the residue using 1 ml HPLC grade methanol. The analysis procedure used for quantification of amount of drug was similar to the measurement of drug loading using HPLC mentioned earlier. Docetaxel release from the nanoparticles was reported as cumulative drug release with time. All values are quoted as mean ± SD.
UV-vis spectrophotometry

Attachment of protein to the nanoparticles was quantified by measuring optical absorbance of the supernatant containing unreacted transferrin before and after the reaction. Absorbance was measured at 280 nm with UV-vis spectrophotometer (Nanodrop-1000) to get protein concentration. Net amount of protein in the supernatant before and after reaction was calculated by protein concentration multiplied by volume.

Protein attached to nanoparticles was then calculated as,

$$\text{protein attached (\%)} = \left( \frac{\text{mass of protein (before reaction} - \text{after reaction)}}{\text{mass(nanoparticles)}} \right) \times 100$$

All values are quoted as mean ± SD.
3.9.3 *In vitro* analysis

**Cytotoxicity assay**

PC3 cells (CRL-1435™, ATCC) were grown overnight in a 12 well plates at a density of 50,000 cells per well using 2 ml of F-12K culture medium (GIBCO®) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% FBS in each well. On the day of experiment, cells were washed with pre-warmed Dulbecco’s phosphate buffered saline (DPBS) and incubated with fresh medium containing docetaxel (dissolved in 0.1% DMSO), Dtxl-Nps and Tf-Dtxl-Nps at equivalent concentration of docetaxel. Control wells were incubated with 0.1% DMSO, nanoparticles without docetaxel loading (Nps) and transferrin attached nanoparticles without docetaxel loading (Tf-Nps). Cells were incubated for 18 h at 37 °C, 5% CO₂. They were then washed thrice with DPBS and incubated in fresh normal medium for a total of 72 h. Cell viability was assessed using MTS reagent. 500 µl of MTS reagent was added into each well and incubated for 3 h at 37 °C. Absorption was read at 492 nm using an Enspire 2300 multimodal plate reader (Perkin Elmer). Experiments were conducted in triplicate, and one-way ANOVA with Tukey’s test at 95% confidence level was applied post hoc to compare unpaired means of absorbance values (n=3).

**Flow cytometry analysis for transferrin receptor expression**

Cells were harvested and counted to make two sets of 5 x 10⁵ cells in triplicate. These samples were then washed (x1) with FACS buffer (PBS with 2% FBS, pH 7.4) and resuspended in 45 µl of FACS buffer. 5 µl of allophycocyanin (APC) tagged anti-human CD71 antibody (anti-CD71) (Affymetrix eBioscience) and APC tagged mouse IgG1 K isotype control (Affymetrix eBioscience) were added and mixed in with samples from each of the two sets respectively. Samples were incubated at 4 °C in the dark for 30 min. Post incubation, excess antibody was washed out twice with 500 µl FACS buffer and centrifuged at 300g for 5 min each. Cells were resuspended in 200 µl FACS Buffer and kept on ice till analyses. Analysis was done using BD LSRFortessa™ SORP cell analyzer (BD Biosciences) within 1 h of sample preparation. APC fluorescence was detected using a 660/20 band pass filter. A total of 80,000 events were recorded and further gated for single cells that were analyzed for TfR expression. Data analysis was done using FlowJo analysis software.
Live cell confocal time lapse imaging

PC3 cells at a density of $4 \times 10^4$ cells/ml (200 µl) were plated into specialized cell culture dishes (MatTek, glass bottom dish; 35 mm dish diameter, 10 mm glass bottom) Cells were seeded and incubated for 60 min to allow them to settle onto the glass coverslip. Dishes were then topped up with additional 2.3 ml of complete F-12K medium (F-12K supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% FBS) and incubated at 37°C in 5% CO$_2$ for 24 h. For imaging, the dish was transferred to an incubator attached to the microscope stage (Tokai Hit, stage top incubator, 37°C, 5% CO$_2$) and imaged with confocal microscope (Nikon A1RMP). While imaging, F12-K culture medium was replaced with complete F12-K culture medium containing either 10 µg/ml of Dtxl-Nps or Tf-Dtxl-Nps. Imaging frames were then taken every 30 seconds for the first 2 min while doing medium exchange, every 15 seconds for the next 10 min followed by every 10 min for a total 18.5 h. Differential interference contrast optics were used for the bright field images and an excitation/emission wavelength of 561/595 nm was used to detect RhB fluorescence from nanoparticles.

3.9.4 In vivo efficacy study

All animal studies for this work were conducted at ProQinase GmbH test facilities (ProQinase GmbH Freiburg, Breisacherstr. 117, 79106 Freiburg, Germany). The Ethics Committee of Animal Experimentation approved the animal study protocol. The experimental protocol was registered by the regional board Freiburg (Regierungspräsidium Freiburg; G-12/62)

Luciferase expressing PC3 cells

PC3 cells were transduced using plasmid encoding a luciferase-neomycin fusion protein and selected using 1 mg/ml G418 antibiotic. Post-selection PC3-LN (CPQ 189, Proqinase®) cells were grown in DMEM-Hi glucose with phenol red supplement, 10 % FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C and 10% CO$_2$

Tumor implantation

A total of 114, 7-8 week old NMRI nude mice (Crl: NMRI-Foxn1$^{nu}$, Charles River GmbH) weighing 25-30g were used for the study. $3 \times 10^6$ PC3-LN cells in 15 µl of PBS were orthotopically implanted into the prostate of each of the participating mice after
anaesthetization. Growth of the implanted tumors was monitored using *in vivo* bioluminescence imaging (BLI) on days 8, 15, 22, 29 and 35. After 22 days of tumor development, animals were randomized into various test groups. On the 24\textsuperscript{th} day after implantation, treatment was initiated with first injection.

*Treatment*

Anti-tumoral efficacy of the three formulations; clinical docetaxel formulation, Dtxl-Nps and Tf-Dtxl-Nps were tested at drug concentrations of 2 mg/kg, 5 mg/kg and 12 mg/kg, respectively. Clinical formulation vehicle (NaCl), Nps and Tf-Nps at equivalent concentrations were injected into control groups. All compounds and vehicle administrations were delivered intravenously into the tail vein. All animals received single 100 µl treatment on days 24, 28 and 32 (one injection every four days for 12 days). Animal weights were monitored every other day and animal behavior was monitored daily. BLI was performed on days 8, 15, 22, 29 and 35. The study was terminated on day 37 and all animals were sacrificed by cervical dislocation. Liver, kidney, primary tumor and spleen were collected for analysis.

*Bioluminescence Imaging*

2 mg D-luciferin was injected intraperitoneally into mice 7 min before anaesthetization. Light emission was measured 10 min post injection with CCD-camera for 1-5 min using a NightOWL LB 981 bioluminescence imaging system (Berthold Technologies, Germany)

*Relaxometry studies*

Nanoparticle samples were diluted to obtain various concentrations for relaxivity measurements. Animal tissues were stored at -80 °C and thawed just before sample preparation for *ex vivo* relaxometry studies. All tissues (liver, kidney and spleen; n=5 each) were weighed (wet mass) and relaxometry measurements were conducted on a 1.4 T Minispec mq series instrument (Bruker). A Carl-Purcell-Meiboom-Gill spin echo sequence was used to measure the proton transverse (R2) relaxation rate with an echo spacing (TE) of 1 ms (2000 echoes) and a repetition time of 10 s. A bi-exponential equation was fitted to the signal intensity data and the fast R2 component is reported in the chapter.
Iron content analysis

Tissues used for relaxometry study were lyophilized for 48 h and their dry mass was determined. Dry tissue samples and nanoparticle solutions were acid digested (99% nitric acid) over 6 h at 95 °C. Reduced samples were diluted with known volume of deionized water and iron content was determined using ICP-AES (inductively coupled plasma atomic emission spectrometry). Iron content for nanoparticles determined using ICP-AES is reported as mean ± SEM (n=4).

3.9.5 Statistical analysis

Results are reported as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) as and where reported in text and figures. ROUT outlier test (Q = 10%) was performed to exclude values if any that markedly deviated from other observations in the sample/group. Statistical comparisons between multiple groups were made using one-way ANOVA ($p < 0.05$), with Tukey’s (corrected for multiple comparison) post-hoc for multiple comparison of means. P values $< 0.05$ were considered significant. Statistical analyses were performed using GraphPad Prism 6.0.
The work presented in this thesis is a progression of the design and utility of PGMA based multifunctional nanoparticles and demonstrates their potential for clinical applications in targeted cancer therapy and imaging. The nanoparticles synthesized herein were aimed at addressing the limitation of current clinical nanoparticle-based formulations that rely on passive uptake mechanisms. The EPR mechanism by itself may not be sufficient for either the therapeutic localization or overcoming the hindrance to diffusivity of macromolecules into solid tumors. Additionally, with the capacity of consolidating multiple features into one construct, these novel second generation nanoparticles are designed to accomplish multiple tasks, including specific delivery to cancer cells, sustained drug release, magnetic resonance and optical imaging, and disease diagnostics. Furthermore, given the versatility of materials used for the synthesis of these targeted nanoparticles, there is immense scope for the future development and extension of application of these nanoparticles in the ever expanding field of cancer theranostics. In this chapter, the findings of the work in chapters two and three will be discussed, perspectives on how the current work may be improved will be outlined and some thoughts in direction of future extension of the targeted multifunctional nanoparticles for application in the field of anticancer therapy shall be put forward.

4.1 PGMA nanoparticles as versatile platforms for ‘click’ assisted folate functionalization and receptor targeting

One of the most attractive aspects of the PGMA nanoparticles that forms the basis of work done in this thesis is the versatility they present for surface functionalization. The availability of epoxide groups on the PGMA molecule provides an advantage over many other polymeric systems in regards to ease of bioconjugation. Most ligand
attachments are reducible to just a one-step reaction via the epoxy ring opening addition reactions, as illustrated in chapter 3 for the attachment of the targeting protein ligand, transferrin. However, some targeting ligands may be deficient in chemically reactive functional groups, sensitive to degradation in non-physiological conditions and on exposure to heat and light, or may require a particular orientation for their biological recognition. For conjugation of these biomolecules, Chapter 2 of the thesis presents PGMA nanoparticles as facile platforms for simple, selective, benign and cost effective surface reactions using ‘click chemistry’.

In this work, an extensively investigated targeting ligand, folic acid was efficiently attached to the PGMA nanoparticles using a simple two-step procedure. First, the nucleophilic ring opening reaction of PGMA to introduce a terminal alkyne using propargylamine, and second, a CuAAC reaction with the azide functionalized FA to finally result in FA attached, RhB labelled PGMA nanoparticles. These FR targeting nanoparticles were confirmed for their biocompatibility in both normal human liver cells, and ovarian and lung cancer cells using the MTS assay. Finally, they were validated for targeting capabilities by their higher uptake in FRα overexpressing SKOV-3 cells in comparison with FRα deficient A549 cells. Non-functionalized nanoparticles did not demonstrate such differential uptake in the two cell lines, suggesting that the enhanced uptake of FA-attached PGMA nanoparticles was FRα-mediated.

The conjugation approach adopted here is promising for its potential to overcome the limitations of the traditionally used methods such as carbodiimide coupling chemistry. Click chemistry provides better control of ligand density, permits reaction progress using mild aqueous conditions, and ensures nanoparticle stability without the generation of any by-products that may require downstream purification. Furthermore, the presence of epoxide groups simplified the acetylation of the nanoparticles, a prerequisite to make the nanoparticles amenable to the ‘click’ reaction. Although herein the application demonstrates FA attachment, this ‘click’-facilitated conjugation approach is generalizable to facilitate an efficient and cost effective surface conjugation of other ligands onto PGMA nanoparticles using mild aqueous conditions and ambient temperature.

The demonstrated selective translocation of FA attached PGMA nanoparticles in vitro in ovarian cancer cells establishes a foundation for future in vivo extension of this work in an ovarian cancer mouse model. However, considering their future application,
PEGylation of the PGMA nanoparticles would be an advantageous enhancement. In particular, a PEG spacer linking the polymeric nanoparticle and the FA targeting moiety would possibly increase the nanoparticle colloidal stability, their systemic circulation half-life, and flexibility of the attached ligand, as well as improve the biodistribution profile of the nanoparticles by lowering their hepatic uptake.

A positive correlation between targeting ligand density on nanoparticle surface and the resulting cellular uptake is well known. However, this correlation is valid only until an optimal intermediate ligand density for which the nanoparticle-cellular interaction is most efficient. Prior to future in vivo translation, it may be prudent to determine the optimal ligand density for the folate-conjugated PGMA nanoparticles to avail maximum in vivo selectivity and by implication, beneficial efficacy and cost effectiveness.

In vivo translation would also require an appropriate imaging modality to be incorporated into the construct to provide in situ information on the localization of the nanoparticles. In the present work, covalent attachment of the fluorescent organic dye RhB facilitated the optical (confocal laser) imaging of the cellular uptake of these nanoparticles. However, for animal studies, it would be better to incorporate a relevant near infra-red (NIR) fluorophore. Organic fluorophores like RhB have lower quantum yield in aqueous environments and are susceptible to photobleaching. This limits the fluence rate that can be applied to the sample and hence the sensitivity of detection. NIR dyes have relatively higher excitation at lower energies, better signal to noise ratio and improved tissue penetration for in vivo imaging. In addition, integration of multimodality into the construct would also be beneficial. An MRI agent like a T2 weighted contrast agent such as iron oxide nanoparticles or a T1 weighted agent such as gadolinium-based agents can be incorporated for application as a complementary imaging modality.

Whilst the ability to perform conjugation on the nanoparticle surface post-synthesis is advantageous, especially for expensive, degradable biological ligands, post-synthesis reaction steps and purification by dialysis can lead to the gradual release of the entrapped drug load into the reaction medium. Therefore, intracellularly cleavable and physiologically stable polymer-drug conjugation (discussed later in this chapter) is proposed to be a better alternative to avoid premature loss of drug load.
Some of the other limitations of this work for consideration prior to pre-clinical studies include the presence of physiological levels of folic acid *in vivo* which may compete with the folate-conjugated nanoparticles for the folate receptor, and the possibility of undesirable distribution and retention of FA-attached therapeutic nanoparticles in systemic tissues that normally express the FR.

4.2 Transferrin receptor targeted multifunctional nanoparticles: Evaluation of efficacy in an orthotopic model of PC3 prostate cancer

Most of the aggressive and metastatic malignancies have a tendency to acquire an invariable chemotherapeutic resistance. This results in negligible therapeutic benefit and serious systemic toxicity from conventional chemotherapeutic treatments providing the impetus for development of alternative targeted therapies for these cancer models. Chapter 3 of this thesis demonstrates the development and validation of the transferrin receptor targeted PGMA multifunctional nanoparticles in an orthotopic model of PC3 prostate cancer. PC3 is characteristic of CRPC; the cell line has been associated with an aggressive phenotype and it is deficient for most prostate cancer biomarkers but displays an overexpression of the TfR.

In this work, clinical Dtxl formulation, passively targeted Dtxl-Nps and actively targeted Tf-Dtxl-Nps at drug doses of 2 mg/kg, 5 mg/kg and 12 mg/kg, respectively, were tested and compared for their efficacy in tumor inhibition *in vivo* in an orthotopic model of PC3 prostate cancer. Both the clinical formulation and non-transferrin functionalized nanoparticles did not demonstrate any significant efficacy in tumor inhibition at a low drug dosage. On the other hand, actively targeted nanoparticles demonstrated significant tumor inhibition at the lowest drug dosage used (2 mg/kg) suggesting their receptor mediated selective uptake. The clinical docetaxel formulation was significantly effective at higher drug concentrations, but this was accompanied with a significant loss in animal body weights, an indicator of unacceptable systemic toxicity. Considering the fact that most clinical studies rely on a response criteria of ‘quality of life’ and ‘overall survival’, transferrin tethered PGMA multifunctional nanoparticles developed in this work are promising candidates for future clinical testing. Their relative selectivity of action may result in the reduction of drug dose required for efficacious tumor inhibition, and they hold the potential to improve docetaxel chemotherapeutic index.
Importantly, the study demonstrates that dosage is a pivotal parameter which needs to be taken into account for the assessment of nanoparticle mediated targeting. Whilst the actively targeted nanoparticles maximize the therapeutic benefits and minimize the off-target effects of the passively targeted nanoparticles, the process of nanoparticle mediated active targeting of tumor cells initially relies on their passive accumulation in tumors. The study demonstrated that at lower doses, actively targeted nanoparticles have distinctly higher efficacy in tumor inhibition than their passively targeted counterparts. However, the EPR effect of the tumor tissue becomes the dominant factor influencing the efficacy of both passively and actively targeted nanoparticles when they are administered at higher doses.

There is potential for both the extension of this work and application of transferrin tethered multifunctional nanoparticles in the treatment of other malignant conditions, such as glioblastoma. Widely used chemotherapeutic agents such as docetaxel, doxorubicin, topotecan etc. are unable to penetrate the blood brain barrier (BBB) which protects the central nervous system and maintains its homeostasis. However, the over expression of the transferrin receptor on brain microvascular or capillary endothelial cells is well documented and has been widely investigated to enhance the uptake of transferrin tethered nanoparticles via receptor mediated endocytosis. The presence of a large number of epoxide functionalities on PGMA nanoparticles can facilitate the incorporation of dual (or multi)-targeting ligands to further achieve cancer cell selectivity, post transferrin mediated BBB penetration, alongside sustained release of drug and MR imaging.

To extend the clinical scope of transferrin tethered multifunctional nanoparticles, which were successfully validated in a pre-clinical model in this work, certain improvements with respect to their physicochemical design parameters could be made as discussed next.

Unlike albumin which is likely to have repulsive protein-protein interactions, the protein transferrin is a cooperative binding protein (Hill coefficient n>1). This suggests that presence of transferrin on a nanoparticle surface is relatively more susceptible to binding of other serum proteins and formation of a protein corona. The corona formation introduces variations in nanoparticle surface characteristics and has recently been reported to push transferrin tethered nanoparticles off target. Further, the corona formation leads to higher reticuloendothelial clearance of the nanoparticles.
as indicated by relaxometry studies performed in this work. For this reason, incorporation of a PEG linker between Tfn and the nanoparticle would introduce stealth properties and provide better orientation flexibility to the ligand.

In addition to the natural ligand, alternative targeting agents have also been identified to drive cellular internalization via binding to the transferrin receptor. These include various biomolecules, such as antibodies, peptides and more recently aptamers which have been shown to perform many folds better than the endogenous protein in competitive binding experiments. Future investigations could possibly involve these biomolecules as alternative targeting ligands to be tethered onto multifunctional nanoparticles for the targeted therapy of Tfr overexpressing cancers.

The multifunctional nanoparticles used in this work were incorporated with an organic fluorophore RhB to facilitate optical microscopy imaging and iron oxide nanoparticles for \textit{ex vivo} MRI imaging. The rate of decay of spin echo recoverable proton transverse magnetization (R2) within tissues was used as a surrogate indicator of nanoparticle concentration because the presence of magnetite nanoparticles in organs is associated with elevated tissue R2 values. R2 values for kidney, spleen, liver and tumor for Dtxl-Nps, Tf-Dtxl-Nps, Dtxl and NaCl treated animals were compared. This \textit{ex vivo} assessment of nanoparticle distribution in the reticuloendothelial system organs suggests a concentration dependent, predominant hepatic clearance of the nanoparticles. The sensitivity of R2 for indication of concentration of nanoparticles in the tumor tissue was insufficient given the natural variation of R2 between different animals owing to various physiological variables. Both the tumor vasculature coupled with dynamic tumor microenvironment change over time and with treatment leading to further variation in R2 values (Appendix D). In future, integration of an NIR dye or agent could provide with an alternative complementary \textit{in vivo} imaging technique alongside bioluminescence imaging of the tumor as is used in this work.

Another factor of critical importance is the preparation of nanoparticle formulation for injection especially for a study design that incorporates drug dosage dependent efficacy analysis. In the present study, nanoparticle formulations with increasing drug doses were prepared by increasing the nanoparticle concentrations and keeping the injection volumes fixed (as nanoparticles have fixed drug loading). However, different nanoparticle concentrations may lead to variation in colloidal stability amongst the injections, which has implication in variability of nanoparticle physicochemical
properties (e.g. size) amongst doses. A better approach for future studies is to adjust the injection volume of the formulation for administration of increasing drug dose, thereby keeping the nanoparticle concentration fixed for a given nanoparticle formulation.

Given their non-biodegradable characteristic, further development of these multifunctional nanoparticles, necessitates a more detailed analysis that facilitates better understanding of their in vivo fate over time, and their persistence at the organ and cellular levels. Present biodistribution studies involved an analysis of only the reticuloendothelial organs, whereas quantitative estimation of the nanoparticles in circulation (i.e. blood) and in vital organs such as the heart is important. Histological examination of normal tissue surrounding the tumor, and tissue collected from other organs would add to the existing knowledge of nanoparticle localization, target specificity and resulting systemic effects.

Most clinical trials on new anticancer agents use a response criteria of overall survival (OS). To be able to translate the pre-clinical data to the clinical setting, a longer observation period following the initial treatment (injection) period is required. This would facilitate survivability analysis over time (Kaplan-Meier plots) which is considered an important aspect to justify further investment in clinical trials.

Apart from the improvement that can be made to each of the individual nanoparticle systems prepared for this thesis, the PGMA multifunctional nanoparticle based platform offers immense scope for future development and extension in other applications in the field of cancer therapeutics as discussed in the following section.

4.3 Extension of targeted PGMA multifunctional nanoparticles in cancer therapy

4.3.1 PGMA multifunctional nanoparticles for combinatorial cancer therapy

Although a single therapeutic agent was used for the work presented in this thesis, the PGMA based nanoparticle system is able to carry multiple payloads through both physical entrapment and chemical conjugation. This can include incorporation of a combination of drugs known to have a synergistic effect or co-delivery of entrapped molecular drug along with either an adsorbed or an attached therapeutic nucleic acid, peptide or an antibody. Such nanoparticles can progressively inhibit tumor growth via cumulative effect of different agents while facilitating a reduction in the required dose of each the drugs, compared to each drug being used alone. This would further imply a
reduction in dose limiting toxicity, suppression of drug resistance etc. For instance, docetaxel and prednisone, the combination of which is clinically approved for metastatic prostate cancer, can be incorporated in a single nanoparticle. Many cancer specific therapeutic antibodies such as trastuzumab and pertuzumab for breast cancer (HER2/ErbB2), cetuximab (EGFR), panitumumab (anti-Her2), and bevacizumab (VEGF) could be attached onto drug loaded PGMA multifunctional nanoparticles to provide them with the ability of targeted combinatorial therapy.

4.3.2 Future direction in polymer-drug conjugation and stimuli responsive intracellular drug release

For the work performed in this thesis, the drug was physically entrapped in the nanoparticles. Docetaxel was incorporated into the nanoparticles by dissolving it in the organic phase along with the polymer for nanoparticle preparation via a single o/w emulsification. The application of this method is limited to hydrophobic drugs (and perhaps hydrophilic drugs that demonstrate pH dependent solubility). Secondly, PGMA nanoparticles have been presented as versatile platform for surface chemistry post nanoparticle synthesis. However, the possibility of premature release of a physically entrapped drug in various reaction conditions may limit the complete utilization of this versatility for chemical conjugation. Finally, the entrapment method usually suffers from low drug loading and there is lack of control on drug release mechanism. The release of an entrapped drug load possibly gets initiated as soon as the formulation is injected into the blood stream in vivo. For this reason, it may be promising to expend future efforts in the direction of polymer-drug conjugation prior to nanoparticle synthesis. This strategy would assist in realization of more efficient drug loading and allow for stimuli responsive intracellular drug cleavage, which would lead to better control of drug release. Polymer-drug conjugation will also assist in extension of PGMA nanoparticles as carriers of both hydrophobic and hydrophilic drugs.

The presence of numerous epoxide functionalities provides with the possibility of introducing various ‘smart’ spacer molecules for the facilitating the attachment and release of chemotherapeutic drugs. A proposed methodology for docetaxel-PGMA conjugate formation is to derivatize docetaxel via its 2’ hydroxyl group (which has been demonstrated to be a suitable site for conjugation) with either levulic acid or glutamic acid (like for XYOTAX™/CT-2103, cleaved intracellularly by cathepsin B).
This docetaxel derivative can then be attached to PGMA polymer via a hydrazide containing spacer e.g. Ma-ah-\(N\)-\(\text{NH}_2\) to form a stimuli-responsive polymer-drug conjugate which would hydrolytically degrade in mild acidic conditions that are prevalent in endosomes (~pH 5). Alternatively the docetaxel derivative can be attached to PGMA via a disulfide containing spacer such as cystamine to form a stimuli-responsive conjugate which would be cleaved by glutathione activity leading to intracellular release of drug. Similarly, methods for attachment of other drugs to PGMA can be also developed.

4.3.3 PGMA multifunctional nanoparticles for targeted delivery of macromolecular therapeutic agents: non-viral transfection and interference therapy

The multifunctional nanoparticles developed in this thesis were used as carriers of a chemotherapeutic drug for cancer therapy. However, with the advent of more personalized molecular medicine they can simultaneously be utilized for targeted delivery of nucleic acids, peptides, miRNA and siRNA for gene therapy and an interference mode of cancer treatment. Non-targeted, cationic (PEI attached) PGMA nanoparticles have been used for shRNA delivery with demonstrated tumor inhibition in \textit{in vivo} models of breast and colon therapy.\(^{150}\) However, PGMA nanoparticles offer the versatility for attachment of both the targeting agent and a therapeutic nucleic acid (or peptide) attachment onto drug loaded nanoparticles. Some techniques proposed for synthesis of such nanoparticles are as follows,

1) \textit{Supramolecular (host-guest) chemistry}: PGMA nanoparticles can be easily conjugated with ethylene-diamine conjugated cationic \(\beta\)-cycloextrin via an epoxide ring opening reaction. The cationic surface could assist with nucleic acid or peptide attachment through electrostatic interactions. Further, if a small hydrophobic molecule (such as cholesterol, adamantane, azobenzene or ferrocene) is attached to the targeting ligand, the ligand can form a host-guest complex with the cycloextrin tethered nanoparticle. Alternatively, attachment of \(\beta\)-cycloextrin to the nanoparticle can be followed by binding both the targeting ligand and nucleic acid/peptide to it, using small molecules forming a host-guest complex. The use of azobenzene as the small connecting molecule, would additionally assist in UV light triggered release of the attached cargo.
2.) Biotin-avidin attachment: Avidin or Neutravidin can be easily immobilized onto PGMA nanoparticle surface via the epoxide ring opening reaction. This makes the nanoparticle surface amenable to attachment of both biotinylated targeting ligands along with biotin attached PEI or biotin attached cystamine. PEI or cystamine can then be utilized for nucleic acid or peptide adsorption.

3) Layer-by-layer assembly (LbL): LbL assembly is a very facile method given the wide choice of poly-electrolytes that can be used for delivery of both cationic and anionic molecules. For e.g. PEI coated PGMA nanoparticles can be layered up with therapeutic anionic molecules like nucleic acids or peptides. For adsorption of cationic peptides or electrolytes like poly(allylamine) hydrochloride (PAH), PGMA nanoparticles can be first coated with poly(acrylic acid) or poly(sulfo styrene) (PAA/PSS). These alternate layers of charged polyelectrolytes, nucleic acid or peptides can finally be followed up with adsorption of charged molecules like hyaluronic acid (which also acts as a targeting molecule) or an alternate polymeric charge reversal layer conjugated to a targeting agent, on the outer most surface.

Further advantage of the PGMA multifunctional nanoparticles for application as transfection agents is the possibility of their extension for enhanced and targeted delivery using ‘magnetofection’. The multifunctional nanoparticles contain superparamagnetic iron oxide nanoparticles. This makes it feasible to use an external magnetic field to direct the transfection of nanoparticles into desired cells and tissues. The advantage of magnetofection over other strategies such as electroporation is that, magnetofection is not limited to in vitro experiments and the concept has the potential to translate to in vivo and clinical applications.

4.3.4 Synthesis of biodegradable PGMA copolymers

Realization of reduction in systemic toxicity of drugs while maintaining their efficacy in the tumor tissue is the primary goal of using nanoparticle based cancer therapeutics. However, it is equally important to confirm that the carrier itself does not impose any health risks to the patient. Biocompatibility is a necessary characteristic of all materials intended for biomedical applications. PGMA nanoparticles synthesized for the work presented in this thesis were confirmed to be biocompatible with various cancer and normal human primary and immortalized cell lines. However, the absence of hydrolyzable and enzymatically cleavable links in the PGMA backbone render the
polymer and the nanoparticles synthesized thereof as non-biodegradable. Although non-degradable and benign materials are desirable for long-term implantable medical devices and tissue engineering products, nanoparticles intended for theranostic applications should ideally be cleared from the body after performing the desired function. Opsonization followed by phagocytosis of circulating polymeric nanoparticles is a well-known phenomenon. Nanoparticles that are not degraded by the phagocytes are sequestered in the organs of the RES. Relaxometry results presented in chapter 3 confirm that the PGMA nanoparticles, being above the renal clearance threshold size, were preferably accumulated in the liver and spleen after the i.v. injection. Such accumulation of non-biodegradable nanoparticles over time can eventually lead to toxicity and other side effects. To circumvent such side effects, future work directed at the synthesis of biodegradable PGMA copolymers would be advantageous. Carefully designed biodegradable PGMA copolymers would also benefit the drug release profile. One approach that could be adopted is to synthesize short (lower molecular weight~ 10-20 kDa) PGMA backbones onto which biodegradable polymeric substituents or dendrons could be grafted. The choice of grafted polymer, its molecular weight and grafting density are important parameters that would need to be considered to ensure that the nanoparticles are successfully localized in tumors and are capable of yielding the desired therapeutic effects prior to their degradation.

4.4 Final Remarks

The data and findings presented in this thesis describe the synthesis, characterization, and post-synthesis functionalization of PGMA based multifunctional nanoparticles. These nanoparticles were evaluated and validated for application in receptor-mediated targeted delivery in models of ovarian and prostate cancers, as was set out in the project aims.

These PGMA nanoparticles are an excellent platform for bioconjugation, which is a key requirement for material advancement in biological applications. Versatility of PGMA nanoparticles for simple click chemistry based FA conjugation presents an example of facilitated, clean, efficient and generalizable low ‘cost’ biofunctionalization of polymeric nanoparticles for application in targeted delivery which is unlikely with other polymeric systems. With additional improvements facilitating stealth behavior and therapeutic potential, these nanoparticles have immense scope for future translation into pre-clinical and clinical studies for the treatment of ovarian cancer.
Pre-clinical studies with Tf-Dtxl-Nps for chemotherapeutic intervention in an orthotopic mouse model of PC3 prostate cancer demonstrated the validity of this novel formulation. These nanoparticles have promising potential in overcoming the drawbacks of the current docetaxel clinical formulation which is approved as a first line treatment for metastatic castrate resistant prostate cancer. Use of a targeting ligand and the provision of a sustained drug release profile enabled the nanoparticles to act with specificity such that, (a) the drug dose required for significant efficacy in tumor inhibition is much lower than that of the clinical drug formulation; and (b) effective tumor inhibition is not accompanied with systemic toxic effects that negatively impact on the patient’s general well-being. The generation of these results in a clinically relevant orthotopic mouse model of prostate cancer is of high significance in promoting further development of this formulation for its progress into clinical application.

While the work in this thesis represents initial efforts; it provides a foundation on which future multiple possibilities available with the targeted PGMA multifunctional nanoparticles may be built. With continual global progress in the field of diagnostics, we now have a better understanding and recognition of the heterogeneous nature of tumor tissue and its inter-individual variations. The versatile nature of PGMA based multifunctional nanoparticles presenting the possibility of encapsulation of a variety of payloads alongwith chemical conjugation of diverse targeting ligands, equips these nanoparticles to overcome the ‘one size fits all’ nature of present clinical practices. Furthermore, future integration of various components to improve the ‘biophysicochemical’ properties of nanoparticles in vivo, together with the loading of multiple therapeutic cargo, and stimuli responsive biological efficacy would assist in the extension of these nanoparticles in anticancer chemo- and gene-therapy.

In summary, the elements presented here and the continual research and development of PGMA based multifunctional nanoparticles will hopefully lead to a brighter future in the field of targeted therapeutics. With the breakthrough influence of nanotechnology in cancer medicine and our ever growing knowledge of the hallmarks of cancer, disease pathophysiology and heterogeneity, nanoparticle based theranostics can provide a foundation to the concept of personalized medicine, which is expected to become the mainstay of safer and more efficacious treatment in the future.
Appendices

Appendix A

Standard Curve for Docetaxel

Docetaxel drug loading in nanoparticles was determined by comparison with the following standard curve of docetaxel. The standard curve was prepared for known concentrations of docetaxel dissolved in HPLC grade methanol, using a Waters 2695 HPLC instrument with a Waters 2489 UV/Vis detector using reverse phase isocratic elution through a C18 column (150 x 4.60 mm, 5 µm, 25 ± 5 °C). A detection wavelength of 232 nm was used for docetaxel. The mobile phase constituted methanol: water (70:30; v/v) at a flow rate of 1.5 ml/min over a run time of 12 min. An injection volume of 20 µl was used for each sample in quadruplicate. The area under the curve at retention time ~ 8 min was integrated.

![Standard Curve](image)

\[
y = 2.385 \times 10^7 x + 16074
\]

\[R^2 = 0.9999\]
Appendix B

Superparamagnetic behavior of magnetite nanoparticles was determined using SQuID magnetometry. In the following figure, plot (a) shows hysteresis loop for magnetite nanoparticles at 5K (black) and absence of hysteresis at 300K (grey). Plot (b) shows zero field cooled (grey) and field cooled (black) curves for magnetite nanoparticles. Magnetite nanoparticles and corresponding polymeric nanoparticles encapsulating the magnetite (Figure 3.2 in text) have a specific saturation magnetization of 63.0 emu/g and 8.4 emu/g, respectively.
Appendix C

The transverse relaxation rate (R2) for polymeric nanoparticles containing magnetite at a given (1 mg/ml) concentration was observed to have a linear correlation with pH of the solution.

\[
y = 4.784x - 3.886 \\
R^2=0.9123
\]
Appendix D

Transverse relaxation rate (R2) and iron concentrations in tumors, for animals treated with NaCl, Dtxl, Dtxl-Nps, and Tf-Dtxl-Nps and docetaxel concentration of 2 mg/kg, 5 mg/kg and 12 mg/kg.

The sensitivity of R2 for indication of concentration of nanoparticles in the tumor tissue was insufficient given the natural variation of R2 between different tumors owing to various physiological variables. Both the tumor vasculature coupled with dynamic tumor microenvironment change over time and with treatment leading to variation in R2 values.
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