Novel Formulations for Magnetic-Resonance Imaging Guided Theranostics

By
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A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY
I Ziwei Zhang confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Ziwei Zhang

(Signed)  
21 December 2021  
(Date)
Dedication

To My Dearest, Beloved Family
Acknowledgement

My PhD journey, was embarked from March 2018 at UCL, London, 7779 miles away from my home. It has been a long but creative, energising journey. Not only it’s about the academic skills and knowledge I’ve gained, but the art of logical reasoning, self-awareness, the capabilities to keeping curious, original and open-minded is the priceless treasure that truly valued. Foremost, my PhD journey would have not been accomplished without my two distinguished supervisors, Prof. Gareth R. Williams (School of Pharmacy, UCL) and Dr Gemma-Louise Davies (Department of Chemistry, UCL). Gareth and Gemma, your intelligence, wisdom and attentive guidance have inspired and motivated me to improve my research, as well as my personality. I am sincerely grateful and appreciate all supports you have provided me through the years.

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Abstract

Recent advances in bioimaging, biochemistry and bioinformatics have facilitated the development of personalized and precision medicine. Theranostics, a combination of imaging modalities and therapeutic agents, have garnered increasing attention in this context, thanks to their potential to monitor and control treatment for individual patients. An attractive strategy to achieve this goal involves the development of therapy guided by magnetic resonance imaging (MRI). MRI, possessing a number of benefits including a high degree of soft tissue contrast, low invasiveness, high depth of penetration and good spatial resolution, could offer advanced imaging-guided therapy enabling precise and time-resolved assessment of disease conditions and therapeutic progression.

The goal of this PhD thesis is to develop novel formulations based on polymeric, inorganic or hybrid materials using two pharmaceutical fabrication techniques (electrohydrodynamic atomisation or spray drying), and explore their potential in MRI-guided chemotherapy. Five different types of formulation carrying MRI contrast agents and chemotherapeutic agents were fabricated.

Chapter 3 reports the fabrication of pH-responsive formulations via electrodynamic atomization, loaded with superparamagnetic iron oxide nanoparticles (SPIONs) as contrast agents and the model chemotherapeutic carmofur. These platforms are able to protect the cargo from release acidic conditions representative of the stomach, while at neutral pH the relaxivity is tightly correlated to the extent of drug release.

Chapter 4 describes a series of dual responsive systems with distinct morphology, comprising of pH-responsive Eudragit shells with SPIONs, and thermo-responsive core loading carmofur. The fibres are found to have better thermo-responsive properties compared to microparticles, and the relaxivity display clear linear relationships with drug release data.

Chapter 5 focuses on using spray drying to fabricate nano-in-micro particles based on a synthetic polymer with an upper capital solution temperature. The microparticles encapsulate drug-loaded layered double hydroxide nanosheets, have thermo-sensitive release and relaxivity profile, and in vitro cell studies reveal that the formulations permit synergistic hyperthermia-aided chemotherapy.
Chapter 6 details the preparation and characterization of four gadolinium doped layered double hydroxides to develop theranostic platforms carrying chemotherapeutics with high $T_1$-relaxivity.

In Chapter 7, polydopamine-coated polycaprolactone/poly(lactic-co-glycolic) acid nanofibers are developed via co-axial electrospinning, which are loaded with dug-loaded LDH nanocomposites in the core. *In vitro* studies reflect sustained release of chemotherapeutics, and highly effective cytotoxic effects on tumour cells with the polydopamine coated formulations, which was further enhanced at higher levels of glutathione.
Impact Statement

There is a need to develop diagnostic techniques to report on cytotoxic agent delivery and release information, as well as on local environment changes in vivo in oncology. Reliable drug release and local condition (e.g. temperature, pH) information helps to reduce side effects of cytotoxic agents in patients, and also to guide drug dosing and thus increase therapeutic efficacy. However, conventional methods to achieve this goal in the clinic are usually invasive, time-consuming, and the resulting dosing data are often delayed. Thus, advanced approaches are required to provide information on dosing and local conditions in a more timely and accurate manner, allowing real-time adjustments of dosing to ensure that local concentrations of a drug are kept within the therapeutic window. Such strategies will be particularly helpful in the administration of highly potent chemotherapeutics which are usually highly harmful to normal tissues.

To achieve real-time in vivo monitoring, magnetic resonance imaging (MRI)-based systems combining therapeutics and MRI contrast agents (known as ‘theranostics’) are advantageous because they: (i) allow non-invasive visualization of the local area with high resolution and (ii) can provide quantitative spatial information on the extent of drug release. Utilization of MRI contrast agents enables deep tissue penetration and quantifiable signal intensity. The research in this thesis attempts to produce theranostics to concurrently deliver chemotherapeutic drugs and MRI contrast agents, aiming to provide high contrast imaging while simultaneously quantifying drug release via MRI signals. Key findings of the work include stimuli-responsive nanocomposite fibres and microparticles with which close correlations between proton relaxivity and drug release were observed. Further, inorganic-organic hybrid materials were developed to simultaneously achieve pH and thermo-responsive drug delivery and MRI. Nano-scale theranostics loaded with cytotoxic agents were developed and exhibited high MRI contrast ability. Finally, an alternative core-shell formulation anchored with MRI contrast agents on the surface was produced as a potential theranostics implant for local release of chemotherapeutics.

The new knowledge gained in this study will contribute significantly to further research to produce MRI-based theranostics by incorporating small molecule chemotherapeutics and inorganic MRI contrast agents into polymeric matrices using facile techniques such
as electrospinning, electrospraying and spray drying. The outcomes of this study will therefore be very informative to researchers seeking to develop novel theranostic systems. In the longer term, they have the potential to provide impact in terms of improved diagnosis and treatment for cancer patients, and ultimately could lead to improvements in human health and wellbeing.
List of Publications

1. Publications resulting from work in this thesis

Research articles

  *This paper forms a part of Chapter 7.*

  *This paper forms the majority of Chapter 6.*

  *This paper forms majority of Chapter 5.*

  *This paper forms the majority of Chapter 4.*

  *This paper forms a part of Chapter 3 and is reproduced with permission from the Royal Society of Chemistry.*

Review

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of Experimental Medicine, National Association of Health Industry and Enterprise Management (PCEM) and John Wiley & Sons Australia, Ltd.

Poster


- Ziwei Zhang, Gemma-Louise Davies, Gareth R. Williams. **Electrosprayed pH-responsive microparticles with encapsulated superparamagnetic nanoparticles and drug for orally delivery theranostics.** 2019 European University Consortium for Pharmaceutical Sciences (ULLA) summer school, University of Helsinki, Helsinki, Finland.

2. Publications resulting from collaborative work outside this thesis

Research articles


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</tr>
</thead>
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<tr>
<td>5FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>APIs</td>
<td>Active pharmaceutical ingredients</td>
</tr>
<tr>
<td>B₀</td>
<td>External magnetic field strength</td>
</tr>
<tr>
<td>B₁</td>
<td>Oscillating electromagnetic field</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>BMA</td>
<td>Bis-methylamide</td>
</tr>
<tr>
<td>BMEA</td>
<td>Bis-methoxiethylamide</td>
</tr>
<tr>
<td>BOPTA</td>
<td>Benzyloxypropionictetracetate</td>
</tr>
<tr>
<td>CA</td>
<td>Contact angle</td>
</tr>
<tr>
<td>CAs</td>
<td>Contrast agents</td>
</tr>
<tr>
<td>CPMG</td>
<td>Carr-Purcell-Meiboom-Gill</td>
</tr>
<tr>
<td>CS</td>
<td>Chitosan</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMAc</td>
<td>N,N-dimethylacetamide</td>
</tr>
<tr>
<td>DMEM-HG</td>
<td>Dulbecco’s modified Eagle with high glucose</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylene-triamine-pentaacetic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Ethyl cellulose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EE</td>
<td>Encapsulation efficiency</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EHDA</td>
<td>Electrohydrodynamic atomization</td>
</tr>
<tr>
<td>EMEA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EOB</td>
<td>Ethoxybenzyl</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeation and retention</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FLI</td>
<td>Optical fluorescence</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HP-DO$_3$A</td>
<td>1,4,7-tris(carboxymethyl)-10-(2’-hydroxypropyl)-1,4,7,10-tetraazacyclododecane.</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometer</td>
</tr>
<tr>
<td>IDD</td>
<td>Implantable drug delivery</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IS</td>
<td>Inner-sphere</td>
</tr>
<tr>
<td>LC</td>
<td>Loading capacity</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower critical solution temperature</td>
</tr>
<tr>
<td>LDHs</td>
<td>Layered double hydroxides</td>
</tr>
<tr>
<td>$M_0$</td>
<td>Net magnetisation</td>
</tr>
<tr>
<td>$M_n$</td>
<td>Molecular number average</td>
</tr>
<tr>
<td>$M_{tr}$</td>
<td>Steady-state transverse magnetization</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Molecular weight average</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>MDSC</td>
<td>Modulated temperature DSC</td>
</tr>
<tr>
<td>MRgFUS</td>
<td>Magnetic resonance–guided focused ultrasound</td>
</tr>
<tr>
<td>MHT</td>
<td>Mild hyperthermia</td>
</tr>
<tr>
<td>MNCs</td>
<td>Mn–Zn ferrite particles</td>
</tr>
<tr>
<td>MNPs</td>
<td>Magnetic iron oxide nanoparticles</td>
</tr>
<tr>
<td>MPS</td>
<td>Monocyte phagocyte system</td>
</tr>
<tr>
<td>MPTT</td>
<td>Mild photothermal treatment</td>
</tr>
<tr>
<td>MRA</td>
<td>Magnetic resonance angiography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MSNs</td>
<td>Mesoporous silica particles</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>$M_z$</td>
<td>Magnetization component along the z-axis</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OS</td>
<td>Outer sphere</td>
</tr>
<tr>
<td>P(AAm-co-AN)</td>
<td>Poly(acrylamide-co-acrylonitrile)</td>
</tr>
<tr>
<td>PAAm</td>
<td>Poly(acrylamide)</td>
</tr>
<tr>
<td>PAI</td>
<td>Photoacoustic Imaging</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PDA</td>
<td>Polydopamine</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDEAAm</td>
<td>Poly(N,N-diethylacrylamide)</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
</tbody>
</table>

*Introduction*
Introduction

PET  Positron emission tomography
PLGA  Poly(D,L-lactide-co-glycolic)
PNIPAM  Poly(N-isopropylacrylamide)
Poly(HPMA)  Poly(N-(2-hydroxypropyl)methacrylamide)
PPy  Polypyrrole
PTT  Photothermal therapy
PTX  Paclitaxel
PVP  Polyvinylpyrrolidone
q  Coordination sphere hydration
r  Proton relaxivity
r_1  Longitudinal relaxivity
r_2  Transverse relaxivity
R_f  Radio frequency pulse
RGD  Arg-Gly-Asp
ROS  reactive oxygen species
rpm  Round per minute
RT  Radiotherapy
SBM  Solomon-Bloembergen-Morgan
S.D.  Standard Deviation
SEM  Scanning electron microscopy
SPAuNC  Superparamagnetic gold nanoparticle
SPECT  Single photon emission computed tomography
SPIONs  Superparamagnetic iron oxide nanoparticles
SS  Second sphere
T_1  Longitudinal relaxation time
T_1e  Spin relaxation time of the electrons
T_2  Transverse relaxation time
Introduction

\textit{TE} \quad \text{Echo time}

\textit{TEM} \quad \text{Transmission electron microscopy}

\textit{T}_g \quad \text{Glass transition temperature}

\textit{TGA} \quad \text{Thermogravimetric analysis}

\textit{TNBC} \quad \text{Triple-negative breast cancer}

\textit{TR} \quad \text{Repetition Time}

\textit{TRP} \quad \text{Thermo-responsive polymer}

\textit{UCST} \quad \text{Upper critical solution temperature}

\textit{US} \quad \text{Ultrasound}

\textit{WHO} \quad \text{World Health Organization}

\textit{XPS} \quad \text{X-ray photoelectron spectroscopy}

\textit{XRD} \quad \text{X-ray diffraction}

\gamma \quad \text{Nucleus}

\omega \quad \text{Angular velocity}

\tau_m \quad \text{Water residence time}

\tau_R \quad \text{Rotational correlation time}

\tau_c \quad \text{Overall correlation lifetime}

\mathcal{D} \quad \text{Molecular weight dispersity}
Chapter 1

Introduction
1.1. General Introduction

Although many advanced therapies have emerged in recent decades, cancer remains a big threat to human health. According to the American Cancer Society, cancer is the second-leading cause of death among children (1-14 years), only next to accidents in 2021 [1]. The corresponding social and economic burdens are considerable. Among more than 100 different types of cancer, an underlying feature is that they are an outcome of some genetic alterations in a single cell, and characterized by uncontrollable cell growth and proliferation [2]. This leads to the outgrowth of abnormal tissues, known as primary tumours. Often, some tumour cells emerging at the original location are able to invade surrounding tissues and move to other parts of the body via the bloodstream. These migrating cells can settle in a new location and grow into secondary tumours, which usually is fatal [3]. It should be noted that not all types of cancer emerge in the form of an outgrowth or lump of tissue. For example, leukaemias, cancers of the blood system, feature uncontrollable growth of cells in the blood, e.g. white cells and plasma cells [4].

Increasing understanding of molecular and tumour biology have made a profound difference in anti-cancer therapy paradigms during recent decades [3]. Formerly, cancer was classified and treated only based on the original sites or simplistic histomorphological characteristics. Now it became clear that the development of molecularly targeted therapies and treatment selection based on particular molecular alterations was needed [5]. As a result, precision oncology appeared as a replacement to the conventional ‘one-size-fits-all’ treatment. Precision oncology involves tailoring personalized therapies to subsets of cancers harbouring specific genomic abnormalities across different tumour types, or targeting components of the tumour microenvironment, in particular the immune system and the antitumour immunity [6, 7].

1.1.1. Anti-cancer Theranostics

The ultimate goal of precision oncology is maximizing therapeutic efficacy while minimizing side effects. One strategy to reach this goal is theranostics-based. ‘Theranostics’ refers to the combination of diagnosis and therapy. Ideal theranostic systems would target specific sites, selectively killing tumour cells, while also enabling non-invasive assessment of disease progression, and quantification of localized carrier
accumulation and drug release [8]. Hence, theranostics should be able to enhance the pharmacokinetics, pharmacodynamics, and biodistribution of an active agent, enabling improvement of therapeutic effect and minimization of side effects [9].

To appropriately integrate imaging-based diagnosis and therapy, several issues should be addressed. First, functional agents should be combined into a single system for the purpose of avoiding any delay between diagnosis and therapy and to monitor in real-time therapeutic efficacy [10, 11]. Second, materials used for theranostics should be biocompatible, nontoxic, and provide good imaging behaviour in vivo. The third issue relates to the need for theranostic systems to specifically target tumours to improve the diagnostic and therapeutic efficiency. Several targeting strategies have been widely utilized for this purpose [12]. Conjugating ligands, such as antibodies which can recognize specific receptors overexpressed on target tumour cells, have been widely employed to this end, an approach referred as ‘active targeting’ [13]. Recently, biomaterials such as the red blood cell membrane, which is rich in CD47, have also been studied to avoid non-specific tissue accumulation [14, 15]. In contrast to active targeting, another approach exploits the enhanced permeation and retention (EPR) effect. This enables macromolecules and nanomaterials to selectively accumulate in tumour cells via leaky blood vessels around tumours [16, 17].

In the section below, we will briefly introduce commonly explored materials for imaging and therapy, as well as discussing nanomedicine and how it is contributing to the development of theranostics.

1.1.2. Imaging Techniques

The biomedical imaging techniques used in the development of theranostics aim to diagnose cancers and identify the heterogeneity of tumours, as well as to monitor the dynamic disease states of patients. A number of appropriate imaging tools are summarized in Table 1-1, and their advantages and limitations discussed below.
Table 1-1 Overview of biomedical imaging methods [18]

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Clinical applications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Computed tomography (CT)</strong></td>
<td>Unlimited tissue penetration</td>
<td>Radiation risk</td>
<td>Bones and joints; Lung diseases, brain disease, various cancers</td>
</tr>
<tr>
<td></td>
<td>High spatial resolution</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Magnetic resonance imaging (MRI)</strong></td>
<td>High spatial resolution</td>
<td>High cost</td>
<td>Brain, oral, breast, liver, lung, lymphoma, kidney, colorectal cancers; Neuronal diseases, Cardiovascular diseases, Bones and joints</td>
</tr>
<tr>
<td></td>
<td>Non-ionizing/non-radioactive</td>
<td>Time consuming</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unlimited tissue penetration</td>
<td>Low sensitivity</td>
<td></td>
</tr>
<tr>
<td><strong>Single photon emission computed tomography (SPECT)</strong></td>
<td>High sensitivity, Unlimited tissue penetration</td>
<td>Radiation risk</td>
<td>Bone, cardiovascular diseases, neurological diseases, various cancers (brain, oral, breast, stomach, prostate, colorectal)</td>
</tr>
<tr>
<td></td>
<td>High resolution</td>
<td>Low spatial resolution</td>
<td></td>
</tr>
<tr>
<td><strong>Positron emission tomography (PET)</strong></td>
<td>High sensitivity, Unlimited tissue penetration</td>
<td>Radiation risk</td>
<td>Vascular diseases, neurological diseases, various cancers (brain, oral, breast, stomach, prostate, colorectal), pharmacokinetics</td>
</tr>
<tr>
<td></td>
<td>Quantitative</td>
<td>High cost</td>
<td></td>
</tr>
<tr>
<td><strong>Optical fluorescence (FLI)</strong></td>
<td>Multi-colour detection</td>
<td>Low spatial resolution</td>
<td>Skin diseases, eye diseases</td>
</tr>
<tr>
<td></td>
<td>Non-ionizing/non-radioactive</td>
<td>Low tissue penetration</td>
<td></td>
</tr>
<tr>
<td><strong>Ultrasound (US)</strong></td>
<td>Non-ionizing/non-radioactive</td>
<td>Low resolution</td>
<td>Pregnancy, cardiovascular diseases, breast colorectal, pancreatic cancers Thyroid-associated diseases</td>
</tr>
<tr>
<td></td>
<td>Low cost</td>
<td>Limited in organs containing gas or behind bones</td>
<td></td>
</tr>
<tr>
<td><strong>Photoacoustic Imaging (PAI)</strong></td>
<td>High spatial resolution</td>
<td>Low sensitivity</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Non-ionizing/non-radioactive</td>
<td>Low tissue penetration</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Limited in tissues blocked by bones or cavities</td>
<td></td>
</tr>
</tbody>
</table>

Computed tomography (CT) integrates a series of images taken from a narrow beam of X-rays that rapidly rotates around a patient, producing cross-sectional ‘slices’ of the patient’s body [19]. Slices collected in a CT scan will then be digitally resolved to generate a 3D picture of the patient that enables accurate determination of the location of internal structures and of any potential abnormalities such as tumours [20]. Since electron dense structures improve the X-ray attention, CT is very capable for imaging hard tissues such as bones [20]. However, CT imaging of soft tissues or the interface between two adjacent tissues remains challenging. Thus, CT contrast agents are often
used to enhance the absolute CT attenuation, which facilitates the differentiation between normal tissues and tissues of interest when selectively delivered to target sites. The advantages of CT include fast diagnosis, high spatial resolution and unlimited depth penetration, but there is a major drawback related to radiation risk [21, 22].

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) respectively utilize radionuclides and γ-rays for in vivo imaging [19]. The nuclides used in PET include $^{11}$C, $^{13}$N, $^{15}$O, $^{18}$F, $^{64}$Cu, $^{68}$Ga, $^{76}$Br and $^{124}$I (all emit positively charged particles). Radionuclides used in SPECT can directly emit γ-rays, such as $^{123}$I, $^{99}$mTc, $^{67}$Ga and $^{201}$Tl, and relatively longer half-lives compared to those used in PET [23, 24]. Hence, SPECT is more economic and usually applied in long-term studies. Both PET and SPECT are more sensitive than MRI, and PET has the highest sensitivity among all the in vivo imaging techniques. However, they also have potential side effects associated with radiation.

Optical fluorescence (FLI) uses light with a particular wavenumber to activate fluorophores to emit detectable light [19, 25]. In comparison to the above imaging techniques, it is non-invasive, relatively inexpensive and convenient to perform. However, there are problems with limited spatial resolution and deep tissue penetration [25, 26]. Near-infrared (NIR) fluorophores have been explored to overcome some of these issues and improve deep tissue penetration and avoid noise related to scattering and auto-fluorescence. A variety of fluorophores with NIR absorption have been developed such as quantum dots [26, 27], organic dyes [28, 29] and upconversion nanoparticles [26, 30].

Ultrasound (US) refers to a fast, and non-invasive imaging technique based on the phenomenon that, when high-frequency sound waves passing through a body encounter a boundary (e.g between tissues), a portion are reflected back to the probe [19, 31]. The intensities and distances of the sound wave echoes can be employed to locate internal organs and provide information about their structures. US can be employed in real-time imaging, but low signal intensities remain a major issue. It is also difficult to obtain imaging about organs containing gas or behind bones [32, 33].

Magnetic resonance imaging (MRI) is advantageous over many other techniques, and as a result is one of the most widely-used imaging technologies in the clinic. Key
benefits of the technique are its high spatial resolution and unlimited tissue penetration. The theory underpinning MRI will be discussed in Section 1.2.1.

Overall, each imaging technique has advantages, but also intrinsic limitations which are different to overcome. Hence, the combination of two or more modalities has been explored with the aim of combining the favourable properties of different technologies [18]. For example, a hybrid imaging modality, PET/MRI, has received great attention in the fields of oncology, cardiology, and neuroscience [18, 34, 35]. It integrates the high-quality images allowing structural and functional characterization of overall tissues obtained via MRI with extremely detailed information in certain areas of interest from sensitive PET imaging. The later permits benefits such as exploring metabolism and tracking of uniquely labelled cell types or cell receptors [35]. Thus compared to PET or PET-CT scan, the potential radio risks can be reduced that patients received lower ionising radiation dose.

1.1.3. Anti-cancer Therapy

Anti-cancer therapies that have been widely used in the clinic include surgery, chemotherapy and radiotherapy. Surgery is used to remove solid tumours that are easy to locate and access [36]. However, it is not effective for tumours that are hard to detect or have metastasized to multiple tissues. Accordingly, surgery is often combined with radiotherapy, chemotherapy or thermotherapy to enhance the therapeutic effects in the treatment of cancers [37, 38].

Radiotherapy (RT) refers to a treatment where high-energy radiation such as X-rays or gamma-rays (external-beam RT) are employed, or where radionuclides are delivered to tumour sites (internal RT), to kill cancer cells [39, 40]. To improve therapeutic efficacy and avoid off-target effects, targeted delivery systems for radionuclides are currently under development [41, 42].

In chemotherapy, small molecules are employed to kill tumours by interfering with cancer cell growth and proliferation [43]. These cytotoxic agents are non-specific however, and also have effects on healthy cells. This causes myriad unpleasant side effect, and hence a number of targeted delivery platforms such as nanoparticle or antibody-drug conjugate systems have been developed target the payload more specifically to the tumour site [44, 45].
Beyond the mainstay chemo and radiotherapies, researchers and clinicians have explored a wide range of alternatives in their quest to develop more potent and specific interventions. Gene therapy is a method that utilizes therapeutic nucleic acids to alter the transcription or translation of DNA or RNA [46-49]. It works by genetically modifying cells and viral particles to stimulate the immune system to kill tumour cells [50]. A major challenge preventing gene therapy from being applied in the clinic is the ability to deliver the intact nucleic acid into target cells. Nucleic acids are very easily degraded before reaching the tumour site, and are hard to transfect into cells because of their negative charge and large size [46, 47].

Photodynamic therapy (PDT) [51-53] and photothermal therapy (PTT) [46, 54, 55] are other possible methods which can be used to treat cancer. These methods convert light energy to chemical or thermal energy, which is then used to kill cancer cells. In photodynamic therapy, reactive oxygen species (ROS) are generated via light irradiation of photosensitizers such as purpurins. These ROS can induce tumour destruction and anti-tumour immune responses [51-53]. Photothermal therapy converts light to heat, and then applies this heat energy to thermally ablate the cells in a tumour [46, 54, 55]. A variety of agents with high light-to-heat photothermal conversion efficacy have been developed, including silver nanoparticles, carbon nanotubes and graphene derivatives [56, 57]. However, as with chemotherapy, PTT and PDT are non-specific. Thus, to realise the full potential of these therapies, targeted accumulation of PDT or PTT agents in a tumour is crucial [58].

1.1.4. Theranostics in Anti-cancer Therapy

Theranostics deliver therapeutic agents and diagnostic imaging agents simultaneously within the same formulation. They hence have the potential to avoid differences in biodistribution and selectivity that may occur if applying separate imaging and therapeutic agents [59]. Advances in biomedicine, bioimaging and nanotechnology have significantly contributed to the development of theranostics [60, 61].

Nanomedicine can play three major roles in theranostics: as carriers, therapeutic and diagnostic agents [9, 62]. As drug carriers, they can improve the solubilization and dissolution of hydrophobic molecules, enhance accumulation of active agents at the pathological area via passive or active targeting, achieve combination therapy via co-
delivery of multiple functional agents, and provide stimuli-responsive release [63, 64].
A variety of therapeutic nanoparticles have been developed for the treatment of cancer. These can achieve targeted killing via reactive oxygen species, radiation or magnetic hyperthermia [60, 61]. As diagnostic agents, a number of highly efficient nanosized contrast agents have been reported for magnetic MRI, CT, photoacoustic tomography or fluorescence imaging [62, 63]. Multiple nanomedicines have been applied to date, including organic nanoparticles such as liposomes, micelles or dendrimers, and inorganic nanoparticles such as mesoporous silica nanoparticles, layered double hydroxides or superparamagnetic iron oxide nanoparticles [65-67].

1.2. MRI

MRI is a useful non-invasive tool widely applied in the clinic and standing out from a number of alternative imaging tools through its high degree of soft tissue contrast, depth of penetration, and spatial resolution [68, 69]. This imaging technique was first reported in the 1940s and has become a versatile technique with multiple functions, allowing the anatomy and physiology of living subjects to be imaged through the spatial distribution of endogenous proton signal intensity [70].

1.2.1. Theoretical Background

1.2.1.1 Spin Relaxation

Nuclei with odd mass numbers and/or odd atomic numbers, e.g., $^1\text{H}$, $^{13}\text{C}$, $^{15}\text{N}$, $^{19}\text{F}$, can be regarded as microscopic magnets and have an intrinsic angular momentum known as spin. Figure 1-1A gives the underlying mechanisms of nuclei spin relaxation. Upon an external applied magnetic field ($B_0$), the random aligned nuclei spins in either parallel or anti-parallel direction of $B_0$. Some of them will align with $B_0$ (Figure 1-1B), leading to a net magnetisation, $M_0$ [71].
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Figure 1-1 (A) Unaligned nuclei spins (arrows); (B) Nuclei spinning aligning parallel or anti-parallel with external magnetic field ($B_0$).

1.2.1.2 Larmor Precession

Magnetic moments of nuclei are induced by applying a static magnetic field. The motion of a magnetic vector about the axis of external magnetic field $B_0$ is described as Larmor precession, which is a trace of a circular path as shown in Figure 1-2 [72]. The nuclei possess an angular velocity ($\omega$ or $\nu$ Hz), referred to as the Larmor frequency, which is linearly correlated to the external magnetic field strength ($B_0$) and the gyromagnetic ratio of the nucleus ($\gamma$), described in Equation 1-1 [72, 73]. The sign of $\gamma$ represents the direction of motion, which may be clockwise or anticlockwise. Thus, a net magnetisation ($M_0$) aligning with $B_0$ were generated by the spinning nuclei, which know as net magnetisation vector.

Figure 1-2 A schematic representation of the angular velocity of atomic nuclei.

$$\omega = -\gamma \times B_0 \, \text{rad} \cdot \text{s}^{-1}$$  \hspace{1cm} \text{Equation 1-1}
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MRI exploits the hydrogen nucleus $^1$H (i.e., a proton) as a magnetic resonance-sensitive isotope and functions by detecting the relaxation times of protons nuclei in endogenous molecules such as water, lipids and proteins [74]. The signal intensities in MRI differ as a result of variations of molecule concentrations, water diffusion rates and proton relaxation rates, allowing the tissues to be differentiated [74, 75].

In MRI, when a radio frequency pulse ($R_f$, induced by an oscillating electromagnetic field ($B_1$, perpendicular to $B_0$) is applied on these net magnetization vectors, they relax from a position parallel to the +z-axis (the direction of $B_0$) to the transverse position [73]. Two relaxations with different mechanisms, longitudinal and transverse takes place, and the exponential time constants required for the relaxations are referred to $T_1$ and $T_2$, respectively [73].

1.2.1.3 Longitudinal Relaxation

Longitudinal relaxation, (or spin-lattice, Figure 1-3), involves the recovery process of the magnetization component along the z-axis ($M_z$) to its equilibrium population [76].

During $T_1$ relaxation, energy transfer occurs from the protons to the environment, until $M_0$ returns to its initial maximum value (Figure 1-4 and Equation 1-2) [76]. $T_1$ is dependent on the rate of the magnetization vector recovery from $+z$-axis to the position parallel to the static magnetic field.

$$M_z = M_0(1 - e^{-t/T_1})$$  \hspace{1cm} \text{Equation 1-2}
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1.2.1.4 Transverse Relaxation

Figure 1-5 shows transverse relaxation, which arises from the loss of coherence of magnetization in the plane perpendicular to the static magnetic field.

During the \( R_f \) pulse, different local magnetic fields lead to slightly different Larmor frequencies of spins, whereas, after the pulse, the nuclei lose their phase coherence as a result of the energy exchange between spinning magnetic nuclei. They then tend to spin in a random pattern [76, 77].

The \( T_2 \), termed the spin-spin relaxation time, describes how rapidly the local coherence decays to zero, as detailed in Equation 1-3 and Figure 1-6 [76].

\[
M_{xy} = M_0 e^{-t/T_2} \quad \text{Equation 1-3}
\]
1.2.1.5 Contrast Mechanisms in MR Imaging

In an MR imaging system, the relaxation process is repeated by application of a rapid series of spaced Rf pulses. Imaging signals are generated by monitoring the nuclear longitudinal and transverse relaxation times $T_1$ and $T_2$ [78]. Longitudinal and transverse relaxation processes occur simultaneously and independently, and the transverse relaxation time $T_2$ is generally much shorter than the longitudinal relaxation time $T_1$ [74].

The chemical and physical nature of the tissues in the body results in endogenous MRI contrast. Here we will briefly discuss the contrast mechanisms in proton MR imaging and three tissue-specific parameters so as to differentiate pathologic from normal tissue.

Three dominant parameters are responsible for inherent tissue contrast in MRI: the proton density, $T_1$ and $T_2$. The relationship between MRI contrast intensity ($I$) and intrinsic relaxation times ($T_1$ and $T_2$), as well as proton density ($N(H)$) can be explored using the equation presenting steady-state transverse magnetization ($M_{tr}$) in a given Rf. For simplicity, here we take spin-echo imaging as an example, which consists of 90°-pulse, a 180°-pulse, and then an echo (Figure 1-7) [79]. The equations describing MR images intensity are given in Equation 1-4 [80]. The time between the middle of the first the 90° $R_f$ pulse and the peak of the spin echo represents the echo time ($TE$). The repetition time between two 90° $R_f$ pulses refers to $TR$, the repetition time.
Figure 1-7 Illustration of conventional spin-echo pulse sequence [79]

\[ I \propto M_{tr} \propto N(H)e^{-TE/T_2}(1 - e^{-TR/T_1}) \]  \hspace{1cm} \text{Equation 1-4}

Proton Density

Based on \textbf{Equation 1-4}, MR image intensity \( I \) is directly proportional to proton density, \( N(H) \). This is because soft tissue enriched with protons generate a macroscopic magnetization after exposure to a magnetic field \( B_0 \), since their spins aligning in or opposite the direction of magnetic field are in a lower energy state. The induced signal intensity of the tilted magnetization is based on the macroscopic magnetization strength, and the larger magnetization leads to brighter pixel intensity in MR images [75]. The macroscopic magnetization within a voxel of tissue is related to the number of spins (proton density, \( N(H) \)) involved and the local temperature [81]. The likelihood of spins to take up the lower energy state is opposed by thermal motions that favor equalization of the two energy states.

\textit{T}_1 \text{ Contrast}

The proton magnetization transverse to the z-axis builds up by providing energy to the spin system with an \( R_f \) pulse. In the intervals between two radio frequency pulses, longitudinal relaxation occurs as a result of magnetic dipole-dipole interactions of fluctuating magnetic fields between local protons or unpaired electrons. Such local fluctuating fields can substantially restore the original orientation of the magnetization vector along the z-axis. A free moving water molecule usually tumbles too quickly to contribute effectively to this longitudinal relaxation; when the protons are partially bound or motion restricted, the relaxation process can be faster, decreasing \( T_1 \).

When experiencing an \( R_f \) pulse, tissues with short \( T_1 \) can restore their longitudinal magnetization \( (M_z) \) faster after an excitation pulse and should have stronger steady-state transverse magnetization \( (M_{tr}) \) than tissues with long \( T_1 \) according to \textbf{Equation 1-4}, provided that changes in \( T_2 \) and proton density are relatively minimal. Therefore, tissues
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with shorter $T_1$ are likely to be brighter in MR images with dominant $T_1$ contrast (known as $T_1$-weighted images). To maximise the $T_1$ contrast, $TE$ should be adjusted to be less than $T_2$ to minimize $T_2$ influence on the signals, and $TR$ has to be approximately equal to the $T_1$ values [80].

$T_2$ Contrast

It is clear that MR images with $T_2$ dominant contrast ($T_2$-weighed images) can be produced with $TE$ close to or longer than $T_2$ in the areas of interest according to Equation 1-4. The $T_2$ contrast can be further improved by extending the repetition time $TR$ to reduce the $T_1$ modulation of the signal intensity. In contrast to $T_1$, $T_2$ images are featured by the brighter appearance of tissues with $T_2$ and darker appearance of tissues with shorter $T_2$, since $T_2$ associated modulation of image intensity $I$ is a function of the factor $e^{-TR/T_1}$ [80].

1.2.2. MRI Contrast Agents

In many cases, the low endogenous contrast in MR imaging is not sufficient for diagnosis of the pathology of interest. Exogenous contrast agents are required in these circumstances to deliver better MRI images, acting by locally shortening the relaxation times $T_1$ and $T_2$. Enhancement in contrast can be achieved when the targeted area possesses either higher vascularity for the contrast agent or higher affinity than other areas. This is often the case with diseased tissues, e.g. tumours, which differ from healthy tissues in terms of metabolism and are composed of abundant capillaries [82, 83]. This means they can take up contrast agents to a greater extent, resulting in better contrast in the MR images.

1.2.2.1 Proton Relaxivity

The proton relaxivity ($r$), defined by Equation 1-4, relates to the efficiency of the contrast agent, specifically the ability of the paramagnetic atoms in the agents to enhance the relaxation of endogenous protons. $r$ is determined by measuring the change in relaxation rate ($\Delta(1/T_i)$, $i = 1,2$) as a function of the concentration of paramagnetic agent or mental ion (M) [84]. The external magnetic field and the temperature also influence the relaxivity of an MRI contrast agent.
Although most MRI contrast agents have effects on both $T_1$ and $T_2$, they are categorized as $T_1$ (usually paramagnetic complexes) and $T_2$ (mostly superparamagnetic iron oxides) agents according to whether their effects are more pronounced for $T_1$ or $T_2$ [77]. The $T_1$ /$T_2$ contrast mechanism is described in Equation 1-4. Most biological tissues have a natural disparity in their endogenous longitudinal and transverse relaxation times – $T_2$ is often 5 to 10 times shorter than $T_1$. The ratio of $r_2/r_1$ is an important parameter describing the contrast efficiency (Figure 1-8): a ratio of 10 or more indicates greater efficiency as a $T_2$ contrast agent, while a $T_1$ contrast agent usually have a ratio of 1-2 [77, 85].

$$r_i = \frac{\Delta T_i}{[M]} \quad i = 1, 2$$  \hspace{1cm} \text{Equation 1-4}$$

Figure 1-8 Illustration of the $r_2/r_1$ ratio affecting the contrast efficiency. A high ratio of $r_2/r_1$ (more than 10 or more) is characteristic of $T_2$ contrast agents, providing a darker image (negative contrast). A low ratio of $r_2/r_1$ defines $T_1$ contrast agents, giving a clearer and brighter image (positive contrast). Diagram reproduced from [77] with permission. Copyright© 2014 Dove Press.

1.2.3. $T_1$ Contrast Agents

$T_1$ contrast agents are those which predominantly reduce the longitudinal relaxation time, giving rise to a hyperintense signals in $T_1$-weighted images (bright contrast) [77]. Generally, rapid radio frequency pulsing is employed to efficiently acquire images with high resolution, leading to the saturation of the MRI signal [70]. A good $T_1$ contrast agent can relieve such saturation by recovering the longitudinal magnetization between RF pulses without affecting the bulk magnetic susceptibility of nearby tissue compartments [70].
1.2.3.1 Theoretical background

The magnetic moment of the paramagnetic atom(s) in $T_1$ contrast agents engages in dipolar and scalar interactions with nearby water protons when a magnetic field is applied (Figure 1-9) [72, 78]. The relaxation of water protons found in the inner-sphere (IS), second sphere (SS) and outer sphere (OS) all contribute to the overall relaxation, as described in Equation 1-5. $R_{1IS}$ is considered to make the most important contribution to $R_1$, as the water protons in the IS are directly coordinating to the paramagnetic centre, whereas those in the SS or OS hydrate and diffuse near the contrast agents, respectively [70].

![Diagram](image)

**Figure 1-9 Schematic diagram showing a $T_1$ contrast agent in solution. The arrow represents water exchange between inner sphere water protons and the bulk water.**

$$R_1 = R_{1IS} + R_{1OS} + R_{1SS} \quad \text{Equation 1-5}$$

According to the Solomon-Bloembergen-Morgan (SBM) theory [84, 86, 87], the relaxivity $r_I$ is dependent on the electronic properties of the paramagnetic centre, the water residence time ($\tau_m$), rotational correlation time ($\tau_R$), first and second coordination sphere hydration (q), and the ion to water proton distance, in addition to the external magnetic field and temperature. By increasing the q values and $\tau_R$ or optimizing $\tau_m$, enhanced $T_1$ contrast can be realized [70, 72].

Increasing the number of water molecules directly coordinated to a paramagnetic centre (q value) via conformation change is a common strategy for $T_1$ contrast agents [70]. Rotational correlation time ($\tau_R$) is mainly dependent on the overall correlation time,
which governs the dipole-dipole interaction between electron and nucleus. For molecular contrast agents, increasing the size of the molecules can decelerate the overall rotation rate of the contrast agent and thus enhance $\tau_R$. For macromolecular and nanoscale contrast agents, the situation is more complicated, and in addition the overall size of the agent and the rigidity of the linker (e.g. a polymer chain connecting the paramagnetic metal ions with nanoparticles) sometimes should be considered [70].

The proton/water exchange time ($\tau_m$) also can be optimized to increase the relaxivity. The overall correlation lifetime ($\tau_c$), governing the dipole-dipole interaction between electron and nucleus, is mainly dependent on $\tau_m$ (Equation 1-6) [72]. The relaxivity decreases when water exchange is too slow to relax bound water efficiently. However, short water residence times also cause decreased relaxivity if the residence times becomes the dominant correlation time, and leads to an increased relaxation time of the bound water [72].

$$\frac{1}{\tau_e} = \frac{1}{T_{1e}} + \frac{1}{\tau_m} + \frac{1}{\tau_R}$$  
\textit{Equation 1-6}

($T_{1e}$ is the spin relaxation time of the electrons, $\tau_m$ is the residence lifetime of the bound water)

Conventional $T_1$ contrast agents include high-spin paramagnetic metal ions such as gadolinium (Gd$^{3+}$), iron (Fe$^{3+}$), or manganese (Mn$^{2+}$) [85]. Most clinically available $T_1$ contrast agents contain the Gd$^{3+}$ ion which possesses seven unpaired electrons, accelerating spin relaxation [77].

1.2.3.2 Summary of Contrast Agents

Free Gd$^{3+}$ cations are cytotoxic and can be retained in the bone, liver and spleen [77, 88, 89]. Gd$^{3+}$ cations are thus often used after chelation with small molecules, to avoid such toxicity. This kind of molecular $T_1$ agents is typically characterized by a neutral or anionic metal chelate with a chemical formula $[\text{M(H}_2\text{O})(\text{L})]$ or $[\text{M(H}_2\text{O})(\text{L})]^n$. M refers to the paramagnetic Gd$^{3+}$ ion, and L is a macrocyclic or acyclic polyaminopolycarboxylate such as 1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid (DOTA), or diethylene-triamine-pentaacetic acid (DTPA) [90]. These molecular chelates can reduce the chance of toxicity that comes from exposure to free Gd ions [91]. Several Gd contrast agents are used in the clinic in MR imaging (Table 1-2 [77]).
Although Gd-chelates are considered to be safe contrast agents, it has been reported in some rare cases that people with renal disease may suffer severe medical conditions referred to as ‘nephrogenic systemic fibrosis’ after administration of Gd-chelates. Therefore the application of Gd contrast agents should be restricted in patients with renal insufficiency or in a perioperative liver transplantation period, as well as in newborn babies up to 4 weeks old, according to the World Health Organization (WHO) [92].

Table 1-2 Gadolinium-based contrast agents approved for clinical use by the EMEA or FDA [77]

<table>
<thead>
<tr>
<th>Generic name</th>
<th>Chemical or CAS number</th>
<th>Type of agent</th>
<th>Product name</th>
<th>Health agency of approbation a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gadofosveset trisodium</td>
<td>193901-90-5</td>
<td>Blood pool</td>
<td>Ablavar</td>
<td>FDA/EMEA (1)</td>
</tr>
<tr>
<td>Gadoxetate disodium</td>
<td>Gd-EOB-DTPA</td>
<td>Targeting</td>
<td>Eovist (formerly Vasevist)</td>
<td>FDA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Primovist</td>
<td>EMEA (2)</td>
</tr>
<tr>
<td>Gadopentetate dimeglumine</td>
<td>Gd(DTPA)</td>
<td>Nonspecific extracellular</td>
<td>Magnevist</td>
<td>FDA/EMEA (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Magnegita</td>
<td>EMEA (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gado-MRT ratiopharm</td>
<td>EMEA (1)</td>
</tr>
<tr>
<td>Gadodiamide</td>
<td>Gd-DTPA-BMA</td>
<td>Nonspecific extracellular</td>
<td>Omniscan</td>
<td>FDA/EMEA (1)</td>
</tr>
<tr>
<td>Gadoversetamide</td>
<td>Gd-DTPA-BMEA</td>
<td>Nonspecific extracellular</td>
<td>OptiMark</td>
<td>FDA/EMEA (1)</td>
</tr>
<tr>
<td>Gadoterido</td>
<td>Gd-HP-DO3A</td>
<td>Nonspecific extracellular</td>
<td>ProHance</td>
<td>FDA/EMEA (3)</td>
</tr>
<tr>
<td>Gadobenate disodium</td>
<td>Gd-BOPTA</td>
<td>Targeting</td>
<td>MultiHance</td>
<td>FDA/EMEA (2)</td>
</tr>
<tr>
<td>Gadoterate</td>
<td>Gd-DOTA</td>
<td>Nonspecific extracellular</td>
<td>Dotarem</td>
<td>FDA/EMEA (3)</td>
</tr>
<tr>
<td>Gadobutrol</td>
<td>Gd-DO3A-butrol</td>
<td>Nonspecific extracellular</td>
<td>Gadovist</td>
<td>FDA/EMEA (3)</td>
</tr>
</tbody>
</table>

* EMEA classification of the contrast agents in relation to the risk of nephrogenic systemic fibrosis: (1) high risk; (2) medium risk; (3) low risk. Abbreviations: EMEA, European Medicines Agency; FDA, US Food and Drug Administration; BMA, bis-methylamide; BMEA, bis-methoxiethylamide; BOPTA, benzyloxypropionictetraacetate; DOTA, 1,4,7,10-tetraazacyclo-dodecane-1,4,7,10 tetracetic acid; EOB, ethoxybenzyl; DTPA, diethylene-triamine-pentacetic acid; Gd, gadolinium; HP-DO3A, 1,4,7-tris(carboxymethyl)-10-(2'-hydroxypropyl)-1,4,7,10-tetraazacyclododecane.
Due to their low molecular weight, most clinical Gd-based contrast agents are extracellular and non-targeted as a result of partial volume dilution effects. This is because small molecular contrast agents usually display fast extravasation from the vascular space to the interstitium, with a distribution half-life of around 5 min, and are eliminated by the kidneys with an elimination half-life of about 80 min [93]. Their fast distribution and elimination dilutes the contrast ability of such agents and often leads to the failure of targeting contrast in vivo [94].

As a result, macromolecular contrast agents including micelles, dendrimers, polymers and other inorganic nanoparticles have been developed to provide targeting contrast and amplify signal intensities. This kind of contrast agent, with high molecular weight, can avoid diffusion through the vascular epithelium and leakage into the interstitial space [77]. This is particularly helpful in MR angiography (MRA), where conventional contrast agents used often suffer from a narrow time domain. While small molecules only stay for a very short time in the blood, macromolecular contrast agents enable slow elimination from the circulation and thus a larger imaging time window [95]. For example, Gd-chelates complexed to polymers (e.g. polylysine derivatives, dextrans, and polyamidoamines) have been applied in MRA [70, 76]. In addition to improving the pharmacokinetic properties, macromolecular constructs can also improve relaxivity as a result of larger paramagnetic metal surface payloads and rotational correlation times [95].

Another limitation of commercially available $T_1$ contrast agents, paramagnetic chelates in particular, is the fact that their contrast efficiency may decrease at higher magnetic fields. A study showed that the relaxivity of Gd-based systems optimized at fields below 1T gradually decreased when higher magnetic fields were applied [84]. Thus, due to their rapid elimination times, detection sensitivity and toxicity concerns, it is necessary to develop novel macromolecular contrast agents for clinical applications.

### 1.2.4. $T_2$ Contrast Agents

#### 1.2.4.1 Theoretical background

$T_2$ contrast agents (or negative contrast agents) are those which primarily decrease $T_2$ of water protons in the regions they are delivered to, producing $T_2$-weighted images and thus darkening the region of interest [77]. These contrast agents affect the transverse
relaxivity by creating local magnetic field gradients around them. The transverse relaxation time decreases can be induced when water diffuses through the local heterogeneous magnetic fields. Therefore, these contrast agents are also regarded as susceptibility agents because of their better performance in the application of gradient echo sequences [77, 96]. While a close interaction between the water protons and contrast agent are required for $T_1$ contrast effect, $T_2$ contrast can be obtained at a relatively remote distance [97].

A classic type of $T_2$ contrast agents are iron oxide nanoparticles, which possess superparamagnetism under a critical upper size limit [98]. There are two forms of iron oxides generally applied for clinical use: magnetite ($\text{Fe}_3\text{O}_4$) and its oxidized and more stable form maghemite ($\gamma$-$\text{Fe}_2\text{O}_3$) [99]. This is because of their low toxicity to humans, chemical stability under the physiological environment and high magnetic moments [100]. In addition, the NPs possess size-dependent magnetic properties and can be used in biomedical application such as magnetic hyperthermia therapy [101].

1.2.4.2 Summary of Contrast Agents

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Core material</th>
<th>Surface</th>
<th>Core size (nm)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>$r_2$ (mM$^{-1}$s$^{-1}$)</th>
<th>Magnetic field (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferumoxides (Feridex)</td>
<td>Fe$_3$O$_4$-$\gamma$-Fe$_2$O$_3$</td>
<td>Dextran</td>
<td>4.96</td>
<td>$\sim$200</td>
<td>120</td>
<td>1.5</td>
</tr>
<tr>
<td>Ferucarbotran (Resovist)</td>
<td>Fe$_3$O$_4$</td>
<td>Carboxydextran</td>
<td>4.2</td>
<td>more than 50</td>
<td>186</td>
<td>1.5</td>
</tr>
<tr>
<td>Ferumoxtran (Combidex)</td>
<td>Fe$_3$O$_4$</td>
<td>Dextran</td>
<td>5.85</td>
<td>less than 50</td>
<td>65</td>
<td>1.5</td>
</tr>
</tbody>
</table>

There are several kinds of iron oxide nanoparticles currently available in the clinic for oral administration, listed in Table 1-3 [102]. For example, Gastromark® (AMAG Pharmaceuticals, Waltham, MA, USA; Ferumoxsil), a silicone-coated superparamagnetic iron oxide nanoparticle (SPIONs) formulation, is applied in gastrointestinal imaging. In order to prevent aggregation, the SPIONs are usually surface coated. Dextran and carboxydextran also used to stabilize the nanoparticles [103, 104].
1.2.5. Applications

The most commonly used contrast agents are gadolinium chelates, they provide a predominant decrease in $T_1$ and an enhancement in positive contrast [77, 96]. Based on their biodistribution, gadolinium chelates can be applied as intravenous contrast agents (e.g., for liver imaging), intravascular contrast agents (e.g., for blood vessel imaging) and targeted and organ-specific contrast agents [105]. Passive targeting Gd chelates have been developed for vascular, hepatobiliary, and reticuloendothelial imaging [105]. There are also other compounds targeting other organs and specific tumours, but to date these have not been clinically used.

Clinically, SPIONs-based $T_2$ contrast agents are applied in liver diseases imaging since they can be selectively uptaken by Kupffer cells, which can be found in the liver, spleen, and bone marrow [102]. They can also be used for MR angiography. Particles with size <50 nm have been used to image lymph nodes [106].

1.3. Theranostics for MRI-guided therapy

MRI works by detecting the relaxation times of protons in endogenous molecules such as water, lipids and proteins [74]. In many cases exogenous contrast agents (CAs) are required to enhance the quality of MRI images and permit accurate diagnoses to be made. CAs work to improve MRI contrast by affecting the local relaxation behaviour of protons. CAs can be incorporated into theranostics to provide detailed information about drug release and delivery. In 2020, a first-in-human trial was reported using MRI-guided nanoparticle theranostics, which can be used to image brain tumour metastases in patients, as well as quantify the local drug concentration [107]. A follow-on phase II clinical trial is ongoing. This offers strong proof of the potential of MRI-guided nanotheranostics in precision medicine. In this section, we will focus on recent progress made in the engineering of MRI-based theranostics to engender in vivo spatiotemporal tracking of active agents and deliver therapeutic effects with high image contrast (Figure 1-10).
1.3.1. MRI guided drug delivery

MRI has been widely explored in personalized medicine to track the route of drug carriers in vivo and provide structural, functional, and molecular information about the tumour region. Here we will review the latest theranostic systems developed for co-delivery of therapeutic agents and MRI CAs. These have been developed to improve bioavailability, overcome complex delivery barriers, assess therapeutic responses, and alleviate adverse effects.

1.3.1.1 T2-MRI

*Overcoming Biological Barriers*

Physiological and pathological barriers challenge drug delivery. A number of MRI-traceable, targeted, theranostics have been designed to address this issue as well as providing real-time information on delivery route and corresponding therapeutic responses. For example, the blood-brain-barrier (BBB), which protects the brain from harmful pathogens, comprises a major biological barrier for drug delivery to the brain. Israel et al. have outlined various strategies to penetrate or enhance permeability across the BBB using magnetic iron oxide nanoparticles (MNPs) via nonspecific/active magnetic targeting [108]. These strategies are particularly helpful in the treatment of solid tumours in the brain, such as glioblastoma (GBM). Ganipineni and co-workers...
reported poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles loaded with paclitaxel (PTX) and superparamagnetic iron oxide nanoparticles (SPIONs) to treat GBM via magnetic targeting [109]. The BBB at the GBM site was seen to be disrupted based on MRI images, and PLGA nanoparticles could be accumulated in the mouse brain via magnetic treatment in an ex vivo biodistribution study. Efforts have also been made to use magnetic resonance–guided focused ultrasound (MRgFUS) to bypass the BBB. This approach works by using low energy ultrasound to transiently disrupt the BBB, and can precisely target systemically-delivered therapy to specific areas in the central nerve systems. Fan et al. fabricated microbubbles loaded with magnetic-labelled doxorubicin that could be accumulated in the brain tumours of glioma rats with the aid of both of focused ultrasound (US) sonication and magnetic fields [110]. Furthermore, the amount of doxorubicin (DOX) present could be quantified based on the $T_2$-weighted MRI signal, providing an advanced molecular therapy for anti-glioma treatment. A recent trial on patients with amyotrophic lateral sclerosis demonstrated that MRgFUS can safely deliver agents to the target areas of the brain, as monitored by MRI [111].

**Targeted Delivery**

MRI guided theranostics for targeted therapy have been extensively explored, typically to treat cancer cells overexpressing particular receptors. Approaches targeting a single biomarker often reach a cell binding plateau with a surface density below the saturation limit, resulting in a low therapeutic efficiency and increasing risks of off-target effect [112]. Hence, active receptor-targeting and magnetic-targeting strategies have been combined to enhance MRI detection and improve therapeutic efficacy. A magnetic mesoporous silica nanoparticle co-loaded with a redox-responsive DOX prodrug and the Arg-Gly-Asp (RGD) ligand was prepared by Yang and colleagues to improve active-targeting ligand-stimulated endocytosis with the aid of magnetic targeting [113, 114]. The nanocarriers prepared were proven to be enriched at the target site as a result of the combined effect of active ligands and external magnetic targeting. Enhancement in $T_2$-weighted MRI images of a murine model bearing αvβ3-integrin positive HeLa tumours was also seen. As a result, the combined approach led to better therapeutic outcomes than either the magnetic or biomarker-targeting strategy alone. Similar work combining RGD and magnetic targeting was achieved with RGD conjugated magneto-vesicles carrying DOX [114]. An in vivo study in mice showed that the magneto-vesicles exhibited potent therapeutic efficacy because of the synergistic effect of active and
magnetic targeting [114]. Researchers have also proposed a dual-receptor targeting strategy, which involves forming multivalent interactions between the drug delivery vehicle and multiple membrane receptors before the former enters host cells. For example, Shen et al. developed RGD and GX1-peptide conjugated magnetic poly-L-lysine MNPs carrying DOX. It was demonstrated that this dual-ligand MRI probe efficiently binds to vascular endothelial growth factor and integrin receptors, and inhibits the growth of tumours in vivo more effectively than nanoprobes targeting a single receptor [115].

Targeted delivery systems also can be envisaged based on materials sensitive to various stimuli, including endogenous stimuli (e.g. pH- and enzymes), exogenous stimuli (e.g. ultrasound or magnetic) and combinations thereof.[116] For example, our group have prepared magnetic nanofibres for oral administration of the chemotherapeutic agent carmofur, using a pH-responsive polymer [117]. These fibres can protect the SPIONs in the acidic gastric conditions and release them at neutral pHs similar to those in the small intestine and colon. This could ultimately permit targeted and MRI-guided antitumour therapy. Further study showed that carmofur release could be quantified using T2 MR relaxometry [117]. Xu and co-workers fabricated magnetically targeted liposomes carrying SPIONs, quantum dots, and cilengitide for active-targeted treatment of C6 glioma cells [118]. In vivo studies displayed enhanced drug delivery at the tumour, and accurate resection of the tumour site could be achieved with the aid of dual MRI-near infrared fluorescence (NIRF) imaging. In another study, polypeptide nanoparticles loaded with the anticancer agent cisplatin and SPIONs were developed for stimuli-responsive MRI-guided theranostics [119]. This system gave release of cisplatin and Fe²⁺/³⁺ in response to the acidic tumour microenvironment, and an in vivo study displayed selective accumulation in the tumour site by T2-weighted MRI.

1.3.1.2 **T₁-MRI**

Most of the theranostics discussed so far employ MNPs as negative CAs for T₂-weighted MRI. In some cases however negative-contrast MRI does not allow the CA signal to be differentiated from artefacts caused by pathological situations such as internal bleeding or calcification, or the boundaries between air and tissue [120]. To overcome these issues, a number of T₁-weighted MRI guided theranostics have been developed. Liu and colleagues fabricated poly(lactic acid)-based particles carrying gadolinium (Gd) ion
chelating DTPA groups and the chemotherapeutic agent sorafenib, seeking to target tumours overexpressing vascular endothelial growth factor receptor [121]. The contrast ability of Liu’s nanomaterials was markedly improved compared to Magnevist®, a commercially available T₁ CA. In vivo studies revealed antitumour effects significantly better than those observed with free sorafenib. In another study, Ling developed a Gd-based nanoparticle-organic hybrid scaffold in which two chemotherapeutic agents, DOX and 5-fluorouracil (5-FU), were loaded [122]. High cytotoxicity to HeLa cells was observed as a result of synergistic pH-responsive release of DOX and 5-FU. Cellular uptake could be imaged using fluorescence and T₁-weighted MRI imaging.

To avoid the potential nephrotoxicity of Gd-based CAs, ultrasmall SPIONs have explored as positive CAs [123, 124]. The high surface area of these materials can facilitate the interaction of paramagnetic Fe²⁺/³⁺ centres with water protons, thus enhancing relaxation. It is reported that a particle size of approx. 5 nm is optimal to offer a high r₁ and a small r₂/r₁ ratio [125]. Multi-functional theranostics have also been developed via integrating multiple imaging modalities such as dual T₁- and T₂-weighted MRI [126], or MRI combined with CT and NIRF imaging. Another alternative approach to avoid Gd-related nephrotoxicity is to utilize manganese (Mn) based formulations. For example, Shi et al. reported 2D MnO₂ nanosheets carrying a therapeutic siRNA with high loading efficiency and evaluated antitumour efficacy in mice bearing tumours [128]. The nanosheets displayed tumour microenvironment-triggered release of siRNA and permitted effective inhibition of tumour growth in vivo. This could be imaged by T₁-weighted MRI.

1.3.2. MRI-Guided Thermal Therapy

Thermal therapies for cancer treatment have gained increasing traction in recent decades as our understanding of the mechanisms behind heat-induced cell killing has improved, and advancements in technology have permitted controlled and localized heating. Thermal therapy is often preferred over chemotherapy because it can be localised and applied repeatedly in a minimally invasive fashion. A variety of techniques are now available for controlled and targeted heating, including lasers, high intensity focused ultrasound, radiofrequency currents or magnetic hyperthermia induced by MNPs.
MRI-guided thermal therapy is non-invasive, and capable of target identification, real-time monitoring of treatment and closed-loop feedback. The latter can be used to further adjust the energy deposition pattern based on MRI. However, the application of high energy stimuli to cause heating can lead to severe damage of the skin or healthy tissues surrounding the treatment areas. In some cases, the energy source is unable to yield the necessary extent of ablation (e.g. for large volume tumour masses or deep lesions). Signal intensity for imaging can also be a concern, with small lesions often being difficult to image owing to low MRI contrast. Hence, theranostics with high hyperthermia efficiency and powerful contrast potency are required address these issues.

Controlling thermal therapy at the tissue and cellular level can be realized using targeting strategies, usually by exploiting nanoscale systems. Here we will give an overview of the MRI-guided systems which have been explored for different type of thermal therapy.

1.3.2.1 MRI-Guided Magnetic Hyperthermia Therapy

Magnetic hyperthermia therapy is a process whereby iron-based nanoparticles, such as SPIONs, generate heat under an alternating magnetic field arising from the size-dependent superparamagnetic properties of SPIONs. This can be exploited in cancer therapy because tumour cells are more susceptible to heating damage (and indeed other therapeutic interventions) than normal cells when the temperature ranges from 42 to 45 °C. SPIONs have been used in thermotherapy for a range of cancers, including brain [129], breast [130], lung [131] and liver cancers [132]. Since SPIONs are also excellent $T_2$ MRI CAs, they have been extensively studied as theranostics for MRI-guided magnetic hyperthermia therapy.

In favorable cases, SPIONs can be injected directly into the target tumour and are largely retained in it during repeated treatments. However, magnetic hyperthermia therapy suffers from problems with insufficient and uneven heating. When the tumour becomes smaller, the local concentration of SPIONs in required to generate adequate thermal energy for hyperthermia dramatically increases based on a model of heat transfer in tissues [133]. Thus, a number of active targeting agents have been developed to enhance the efficacy of hyperthermia induction, as well as produce high quality MRI images to monitor the magnetic hyperthermia therapy outcome. In one study, Soleymani fabricated folate-targeted iron oxide nanoparticles (FA@Fe$_3$O$_4$ NPs) for simultaneous
MRI imaging and magnetic hyperthermia therapy [134]. The active-targeted nanoparticles could selectively accumulate in the tumour site, as monitored by $T_2$-weighted MRI, and thereby enhance magnetic hyperthermia therapy efficacy.

In addition to SPIONs, other metal hybrid inorganic nanoclusters have also been developed as theranostics for MRI-guided magnetic hyperthermia therapy. For example, a Mn-Zn ferrite magnetic system was synthesized and conjugated with a fluorescent dye and peptide ligands to serve as a RGD receptor-tailed, dual-modal (MRI/fluorescence) agent for magnetic hyperthermia therapy [135]. However, it still remained impossible to provide adequate heating to ablate tumours. This arose due to low particle concentrations in the tumour site, despite the enhanced targeting effect of RGD. In another work, Kwon utilized a viral capsid particle to prepare inorganic nanocluster hybrids consisting of superparamagnetic gold nanoparticles (SPAuNC, **Figure 1-11A**) grafted with peptide ligands targeting the epidermal growth factor receptor-ligands overexpressed on tumour cells [136]. *In vivo* studies revealed that this theranostic could actively target tumour cells and enhance $T_2$-weighted MRI and magnetic hyperthermia therapy (**Figure 1-11B-D**).
1.3.2.2 MRI-Guided Ultrasound Thermal Therapy

Ultrasound (US) is a technique used clinically for both imaging and therapy. It is characterized by its ability to penetrate deep lesions. Recently, temperature-sensitive MRI has been used to guide ultrasound surgery for selective tumour ablation based on calculated results of thermal dose from multislice imaging [137, 138]. MRI-guided ultrasound surgery successfully removed tumours within a planned area in the prostates of both canines and humans using a directional transurethral ultrasound applicator [138].

There is a problem with standard US in that to heat effectively it requires the application of high-energy acoustic waves, which might lead to damage to nearby healthy tissues. As a result, efforts have been undertaken to prepare thermosensitive agents to improve
the heating efficiency. For instance, a thermosensitive magnetic-guided active targeting liposome incorporating fullerene (C$_{60}$), SPIONs and docetaxel (DTX) has been developed [139]. This was found able to act as a radiofrequency thermal therapy agent, which can trigger the release of DTX as observed in $T_2$-weighted MRI and eradicate the cancer cells by heat energy generated from radiofrequency irradiation. A more recent study reported a Gd-labelled thermo-responsive liposome that can be concentrated in a tumour and released most of its DOX payload using a rapid burst of focused US (3 min) [140]. The accumulation of liposomes at the tumour site also caused a reduction of the $T_1$ relaxation time, allowing for theranostic applications.

To improve therapeutic outcomes, MRgFUS has been widely studied as a route to use MRI to guide US-mediated thermo-ablation [131]. In the clinic, the US energy source must be precisely controlled, which can be achieved in MRgFUS by using MRI to provide details of changes at and around the treatment area. For example, gradient echo MR imaging can be used to monitor the temperature in real time during MRgFUS treatment. This is achieved by monitoring the relaxation time and exploiting the temperature sensitivity of the proton resonance frequency. Wang et al. proposed a SPION nanosystem that actively targets EGFR, since the latter was over-expressed in a number of epithelial tumours [131]. This active targeting system led to significant enhancement of MRI sensitivity in rat models. When applying MRgFUS, the SPION-based theranostics could lead to effective ablation of a tumour at a relatively low energy levels.

1.3.2.3 MRI-Guided Photothermal Therapy

Photothermal therapy (PTT) is another minimally-invasive therapeutic approach that has been combined with MRI. PTT works to ablate cancer cells by generating heat energy from electromagnetic radiation. This is generally achieved by using a photo-absorbing agent to absorb near-infrared (NIR) light from a laser and convert this to heat. PTT can be designed to specifically target tumours with spatial and temporal precision [18]. Factors affecting the therapeutic efficacy of PTT include the incident excitation energy (e.g. the light intensity) and the dosage and photothermal conversion efficiency of the PTT agents. A number of PTT agents based on inorganic, organic, carbon, or semiconductor materials have been explored, seeking to prepare systems with (i) high
photothermal conversion efficiency, typically seeking strong absorption of NIR light; (ii) excellent targeting capability; and (iii) low toxicity and high biocompatibility [141].

Since MRI CAs (e.g. Gd-based agents and SPIONs) tend to have low photothermal conversion efficiency, they must be combined with photoactive materials for MRI-guided PTT. One targeted platform for theranostic MRI and fluorescence imaging and PTT was prepared via conjugation of hyaluronic acid and CuInS$_2$-ZnS quantum dots on the surface of a magnetic Prussian blue.[142] In a murine HeLa tumour model, this system exhibited effective uptake in the tumour site using both magnetic and CD44 receptor active targeting. Uptake could be monitored by MRI and NIR fluorescence, and the formulation led to significant temperature rises (up to around 49 °C) and thus had excellent in vivo PTT efficacy.[142]

Magnetic targeting has been used to deliver PTT agents and improve photostability, biodistribution, and pharmacokinetics. For example, indocyanine green (ICG) is a clinically approved photothermal agent for fluorescent-guided surgery but suffers from poor photostability and low bioavailability. Incorporating ICG into nanocarriers increases the circulation time and enhances targeted delivery into tumours [143]. In one study, a magnetic nanocluster consisting of a Fe$_3$O$_4$ core and a polydopamine shell grafted with polyethylene glycol and indocyanine green was fabricated for combined MRI and PTT [144]. The formulation could be magnetically targeted to accumulate in HepG2 liver tumours in a mouse model, and improved the efficacy of PTT because of the presence of two NIR absorbers, (polydopamine and indocyanine green). The therapeutic process could be monitored by $T_2$-weighted MRI and an infrared thermal camera. More recently, polydopamine coated mesoporous silica particles loaded with manganese ions were proven to be potent $T_1$ MRI CAs and photosensitizing agents with excellent photothermal conversion efficiency in an in vivo mice model [145]. A computational model was established to simulate the heat transfer process in the tumour during PTT, based on MRI data and nanoparticle distribution in vivo. This allowed accurate predictions of the in vivo performance [146].

Recent studies have provided a variety of approaches to enhance MRI-guided PTT efficacy, including employing absorbance in the NIR-II window (900 – 1700 nm) to reduce noise arising from autofluorescence and photon scattering, and enhance the penetration depth [147, 148]. Other work sought to combine MRI with other imaging
modalities such as photoacoustic imaging or US [149, 150], or use subcellular targeting strategies to deliver a payload selectively to the nucleus [151]. 1D-ferrous phosphide nanorods were developed by Liu as a multi-modal theranostic for $T_2$ MRI/photoacoustic imaging and enhanced PTT [152]. This formulation allowed PTT (with high PTCE in the NIR II window) to be combined with chemodynamic therapy, providing a promising platform for targeted, controllable, antitumour treatment with simultaneous MRI/PAI.

PTT can result in local tissue temperatures over 50 °C, which can be harmful to healthy surrounding tissues. To avoid this issue, mild photothermal treatment (MPTT) has been devised, seeking to ensure a maximum temperature of around 45 °C. Such low temperatures do not usually result in effective PTT, because the cell damage induced by mild heating stress tends to be repaired by a cell’s intrinsic thermo resistance system with the aid of heat shock proteins. Thus, strategies have been developed to permit effective PTT at lower temperatures [153].

One such approach is to convert oxygen radicals induced by heat stress to highly toxic ROS such as •OH [153]. Moderate hyperthermia can increase the oxygen concentration in cells by reducing oxygen consumption via alternation of cell metabolism pathways and inhibition of cell respiration [154, 155]. In one study, Qiu et al. fabricated magnetic nanoparticle-iridium (III) complexes that actively targeted mitochondria and could efficiently kill tumour cells upon NIR laser irradiation [156]. The nanoparticles were seen to accumulate in the tumour site of mouse models, using $T_2$-weighted MRI images. Cell apoptosis was induced by generation of •OH at a local temperature of 42 °C.

1.3.3. MRI-guided combination therapy

Combination therapies are desirable in a number of disease settings to avoid drug resistance, lower the required dosage, and reduce adverse effects. In this section, MRI-trackable theranostics for multimodal therapy will be outlined.

One major drawback of PTT is the uneven distribution of heat and restricted tissue penetration, which can lead to unsuccessful tumour ablation and a high risk of cancer recurrence [157]. The combination of PTT with other therapeutic approaches such as photodynamic therapy (PDT) [158-160] and chemotherapy [161, 162] has been found helpful to overcome this challenge, particularly for complex, large, and heterogeneous tumours. For instance, an iron-based nanoparticle with $T_1/T_2$ MRI contrast ability has
been developed as a theranostic platform to deliver DOX and conduct PTT [163]. In vivo studies in tumour-bearing mice showed that the system could be accumulated in a tumour as a result of the enhanced permeation and retention (EPR) effect, as well as enhancing both $T_1$ and $T_2$-weighed MRI. Excellent tumour-killing effects were observed as a result of the synergy between chemotherapy and PTT.

Photodynamic therapy (PDT) is a minimally invasive and targeted therapy which works by local generation of highly active ROS. These are formed by energy transferred from light activation of photosensitizers [164, 165]. PDT agents are non-toxic in the dark, which makes the therapeutic process highly controllable. Recently, MRI-trackable agents to combine PDT-PTT therapy have attracted increasing attention. For example, a multifunctional smart nanoplatform was developed for combined PTT-PDT therapy which could be imaged by $T_1$-weighted MRI [166]. The formulation was composed of a tumour-microenvironment sensitive carbon nanotube scaffold loaded with a photosensitizer (Ce6), and MnO$_2$. In the low pH of the TME, Ce6 was rapidly released for PDT, and Mn$^{2+}$ was freed into solution for $T_1$ MRI. The authors showed that MnO$_2$ helped to catalytically decompose the excess H$_2$O$_2$ present in the tumour into O$_2$, which can further promote ROS production and enhance PDT efficacy. Guo and co-workers prepared a MnO$_2$ based nanoenzyme loaded with polypyrrole and evaluated its therapeutic effect as a MRI-guided PTT-PDT agent in tumour-bearing mice [167]. The results showed that the nanoenzyme gave enhanced $T_1$ contrast and outstanding tumour ablation performance. Another PDT-PTT-chemotherapy platform based on black phosphorus nanosheets (BPN) was reported by Wu et al [168]. When decorated with MnO$_2$ nanosheets, the BPN system exhibited enhanced phototherapeutic potency. The time-dependent accumulation of the formulation in the tumour site was clearly shown in $T_1$-weighted MRI images. In another work, Lin et al. reported a Prussian blue nanoparticle grafted with PDA and Ce6, as a multifunctional theranostic for combined PTT and PDT [169]. Both In vivo and in vitro studies confirmed inhibition of tumour growth and moderate enhancement in $T_2$-weighted MRI.

Feng and co-workers fabricated a combined PTT-PDT-chemotherapy theranostic consisting of magnetic mesoporous CuS particles loaded with DOX [170]. When exposed to NIR irradiation DOX release could be triggered, and PTT-PDT combined therapy was achieved via localized surface plasmon resonance and increasing ROS levels caused by the leakage of copper ions. $T_2$ MRI can be used to monitor the resultant
therapeutic effects. Using multiple targeting approaches can further enhance the efficacy of chemotherapy-PTT, while combining this with MRI capabilities enables accurate and real-time imaging of the therapeutic effect. A variety of active targeting platforms have been developed to this end, such as: folate conjugated Ag-Fe₃O₄ particles carrying paclitaxel (PTX) [147]; EGFR-targeted erbitux-modified liposomes loaded with gold nanorods and DOX [162]; PLGA-coated Fe₃O₄ graphene oxide nanomaterials containing 5-fluoruracil (5-FU) [171]; and, polymer hybrid MNPs carrying the photosensitizer IR820 and PTX [161]. All the systems have been shown to be potent in MRI-monitoring of PTT-chemotherapy treatments.

1.4. Stimuli-Responsive Polymers

It is clear from the discussion above that effectively targeting a diagnostic or therapeutic entity is key to achieving the desired patient outcomes. Polymers offer one route to achieve this. Researchers have explored a variety of water-soluble stimuli-responsive polymers to provide spatially and temporally controlled delivery of anti-cancer agents. These systems have properties switchable upon small changes in a stimulus such as temperature, pH or ROS presence. These controlled-release behaviours can provide advantages including reducing dosage and side effects. pH-responsive and thermo-responsive polymers have been most widely explored, and here recent advances are reviewed and a selection of applications are given.

1.4.1. Volume Phase Transition

Polymer gels can exhibit drastic swelling-shrinking behaviour with an infinitesimal alternation of the external environment, e.g. the pH or temperature. Four fundamental interactions between macromolecules which are responsible for volume phase transition can be categorised: ionic, hydrogen bonding, hydrophobic interactions and Van-der-Waals forces [172, 173]. For example, hydrophobic interactions govern the phase transition of poly(N-isopropylacrylamide) (PNIPAM). This undergoes a distinct hydrophobic/hydrophilic transition in water when the temperature changes: at high temperature, the polymer exists in a collapsed state, but when the temperature is decreased, the hydrophobic interactions become weak and the gels transfer to a swollen state. On the contrary, polymer gels with cooperative hydrogen bonding (e.g. an
interpenetrating polymer network of poly(acrylic acid) and poly(acrylamide)), go through a phase transition from swollen to collapsed in water when the temperature decreases to a certain value [174]. In terms of pH-driven phase transitions, attractive ionic forces are the key interaction.

1.4.2. Thermo-Responsive Polymers

Polymers which process a volume phase transition at a certain temperature (the critical point) are thermally responsive. This transition leads to a reversible and sudden transformation in solvation state [175]. The biomedical applications of such polymers is based on the fact that the body-site temperature can shift spontaneously upon local infections or diseases, or be changed intentionally by application of external stimuli such as magnetic hyperthermia [176]. The solubility of a polymer characterized with a lower critical solution temperature (LCST) decreases upon heating [177], while that of polymers having an upper critical solution (UCST) increases upon heating. Here we focus on polymers in an aqueous solvent environment, which is of value in terms of the biomedical applications. Their volume phase transition, with a coil to globule morphology change, is led by transformation in hydration states. This is because of the competing interactions between solvent and polymer, the hydrogen-bonding interactions between polymer and solvent, and the hydrophobic/hydrophilic balance inter- and intra- polymer molecules [178].

Thermodynamically, the phase transition can be explained by the entropic effect of the dissolution process and the ordered state of water molecules in the vicinity of the polymer, and the balance between enthalpic effects caused by intra- and intermolecular interactions (e.g. ionic interactions, hydrogen bonding) and solvation (e.g. hydrogen bonding and hydrophobic interactions). The entropic contributions are believed to be dominant in LCST-type polymers, while enthalpic effects are more important in determining UCST [175]. For example, the solubility of zwitterionic polymers usually remains very low at low temperatures due to the positive and negative charges they bear and the resultant strong ionic interactions within the polymer. Interchain H bonds similarly prevent the dissolution of H-bonded polymers. Thermal activation is required to break down those interaction within the polymers such that they can dissolve, resulting in a typical UCST transition.
It should be pointed out that the terms LCST and UCST are only used when the phase diagram (Figure 1-12) of temperature vs. polymer/solvent composition has been determined and displays both one-phase and a two-phase region. A maximum or minimum temperature can be observed on a phase transition plot for UCST or LCST-type polymers. Otherwise temperatures where to solubility changes occur are regarded as transition temperatures.

Besides temperature, the solubility of these polymers in an aqueous environment is also affected by a variety of other factors, including concentration, molecular weight, and the presence of any additives. Since their thermo-responsive behaviour relies on competing interactions (hydrogen bonding or hydrophobic forces) within the polymer or between polymer molecules and solvent, additives such as salts, co-solvents or surfactants can shift the position of the volume phase transition. These additives can influence the solvent-polymer interaction by changing the solvent properties. Additionally, surfactants, with amphiphilic structures, can affect the hydrophobic/hydrophilic balance of the overall systems through interacting with polymer molecules. For example, by adding sodium dodecyl sulfate, an ionic surfactant,
the hydrodynamic diameter of PNIPAM gradually grows, and the transition temperature also increases [179].

A number of localized in vivo temperature-mediating approaches have been proposed and investigated in clinical settings, for instance near infrared light (NIF), microwaves, ultrasound and magnetic resonance [180, 181]. Hyperthermia with different and heating times has been investigated in clinical trails: at the lower temperature (39-41 °C), the heating times can go up to 72h, while a higher temperature treatment (42-45 °C) utilizes a repeat heating time of around 15-60 min. No harmful side effects were observed in either treatment [181-183].

Compared to normal cells, cancer cells are more sensitive to high temperatures, so hyperthermia-triggered delivery vehicles are especially attractive for local temperature-induced apoptosis in anti-cancer therapy [176, 184, 185].

1.4.3. **UCST or LCST Polymers for Biomedical Applications**

A selection of polymers with either UCST or LCST behaviour suitable for biomedical applications is given in **Table 1-4**. Their transition temperature should lie in the physiological range (20 to 40 °C).
Table 1-4 Examples of UCST and LCST polymers suitable for biomedical applications

<table>
<thead>
<tr>
<th>Type</th>
<th>Polymer</th>
<th>Chemical structures</th>
<th>Transition Temperature in aqueous solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCST</td>
<td>PNIPAM</td>
<td>R₁=H, R₂=Pr; PNIPAM</td>
<td>30-34 °C [186, 187]</td>
</tr>
<tr>
<td></td>
<td>Poly(N,N-diethylacrylamide) (PDEAAm)</td>
<td>R₁=R₂=Et; PDEAAm</td>
<td>32-34 °C [188]</td>
</tr>
<tr>
<td></td>
<td>Poly(methyl vinyl ether)</td>
<td></td>
<td>37 °C [189]</td>
</tr>
<tr>
<td></td>
<td>Poly(N-vinylcaprolactam)</td>
<td></td>
<td>30-50 °C [190, 191]</td>
</tr>
<tr>
<td>UCST</td>
<td>Poly(acrylamide-co-acrylonitrile) P(AAm-co-AN)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Poly(acrylamide) PAAm</td>
<td></td>
<td>25 °C [192-194]</td>
</tr>
<tr>
<td></td>
<td>Poly(acrylic acid) PAAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ureido-derivatives</td>
<td></td>
<td>8-65 °C [195]</td>
</tr>
<tr>
<td></td>
<td>poly(N-(2-hydroxypropyl)methacrylamide-glycoamide) (poly(HPMA-GA))</td>
<td>Transition temperature around 40-50 °C [196]</td>
<td></td>
</tr>
</tbody>
</table>

1.4.4. pH-Responsive Polymers

Increasing attention has also been paid to polymers reacting to pH changes. These polymers contain acidic or basic functional groups, and hence the conformations of their chains undergo a sudden change around the pKₐ due to drastic variation in ionization levels of pendant groups, resulting in a change of water solubility. For biomedical pH-responsive systems, these polymers should have a pKₐ value of 3-10. Two example pH-responsive polymers are given in Figure 1-13. The carboxylic groups of poly(acrylic acid) release protons and become ionized beyond its pKₐ, resulting in increasing water...
solubility. The opposite phenomenon can be observed for polybases, e.g. poly(N,N’-diethylaminoethyl methacrylate). The pH range where the transition occurs can be manipulated by the introduction of hydrophobic components. The corresponding critical pH will change as repulsive electrostatic interactions are required to depress the hydrophobic forces [197].

![Figure 1-13 Examples of protonation and deprotonation processes of polyanions (top) and polycations (bottom), reproduced from [197]. In acidic conditions, poly(acrylic acid), a weak polyacid, stays in the deionized state and thus the polymer is insoluble. Under the same conditions, poly(N,N’-diethylaminoethyl methacrylate), a weak polybase, is ionized and soluble in water. In basic conditions, the opposite behaviour is observed in both polymers.](image)

pH-responsive polymer systems have been widely used in a number of biomedical applications, particularly in the controlled delivery of bioactive agents such as indomethacin [198], carmofur or lysosome [199]. For examples, Eudragit S100 and L100, anionic copolymers based on methacrylic acid and methyl methacrylate, have been widely used for developing oral formulations for delayed release [200]. Due to the acidic environment in the tumour, a variety of pH-responsive delivery systems have been studied for intracellular and extracellular release of bioactive agents [201, 202]. The most commonly utilized strategy to achieve this is via the protonation of polymers.

For example, Cheng, et. al fabricated an intelligent nanocarrier based on a synthetic amphiphilic lignin-related copolymer [203]. This biocompatible system enabled pH-responsive release, retaining most of its ibuprofen cargo at pH 1.5 while controlled releasing it at neutral pH relied on a “switching” mechanism. Another pH-responsive strategy developed by Pujara and co-workers to orally deliver a poorly soluble nutraceutical (curcumin) relied on pH responsive nanocarrier [204]. In vivo studies
showed that the system could protect hydrophobic drugs in acidic conditions and enhance oral bioavailability.

Polymers with ionizable components can thus be used to construct nanocarriers for anti-cancer therapy. In anti-cancer treatment, such carriers can be taken up via the enhanced permeability and retention (EPR) effect. Upon reaching the acidic target, they will undergo conformational transformation or disassembly via destabilisation or precipitation/aggregation (Figure 1-14), resulting in the release of the entrapped agents either extracellular or intracellularly [205].

![Figure 1-14](image)

**Figure 1-14** Illustration of pH-triggered drug delivery at tumour sites. 1: Extracellular release caused by dissociation (pH 6.5), or 2: intracellular release after endocytosis, due to protonation.

**Figure 1-15** gives selected examples of pH-responsive polymers that can be applied for pH-triggered release. Acidic polymers such as poly(acrylic acid) [206, 207], poly(methacrylic acid) [208], poly(2-ethyl acrylic acid) [209], poly(propylacrylic acid) and poly(glycolic acid) are all widely explored, as detailed in Table 1-5 [175, 210]. Polycations (e.g. polyamines, polyethylenemine) have also attracted much attention, for instance in the form of poly(N,N'-diethylaminoethyl methacrylate) [211] and poly(N,N'-dimethylaminoethyl methacrylate) [212] (Table 1-5).
Figure 1-15 Chemical structures of pH-responsive polymers widely explored for biomedical applications
Table 1-5 A selection of pH-responsive polymers explored for biomedical applications

<table>
<thead>
<tr>
<th>Type</th>
<th>Polymer</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyanion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poly(aspartic acid)</td>
<td>4.88 [213]</td>
</tr>
<tr>
<td></td>
<td>Poly(acrylic acid)</td>
<td>4.75 [214, 215]</td>
</tr>
<tr>
<td></td>
<td>Poly(methacrylic acid) and Poly (ethyl acrylic acid)</td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>3-methylglutarylated poly (glycidol)</td>
<td>6.30 [217, 218]</td>
</tr>
<tr>
<td></td>
<td>Poly-sulfonamides</td>
<td>6.8 [219, 220]</td>
</tr>
<tr>
<td><strong>Polycation</strong></td>
<td>Poly(β-amino ester)</td>
<td>6.50 [221]</td>
</tr>
<tr>
<td></td>
<td>poly(N,N’-diethylaminoethyl methacrylate)</td>
<td>7.50 [222, 223]</td>
</tr>
<tr>
<td></td>
<td>Poly(L-histidine)</td>
<td>Around 7.0 [224]</td>
</tr>
<tr>
<td></td>
<td>Chitosan</td>
<td>Around 6.50 [225]</td>
</tr>
</tbody>
</table>
1.5. Nanotechnology

Nanoparticles have attracted intensive attention in biomedical applications [202, 226]. In this section, we introduce two nanoparticles, superparamagnetic iron oxide nanoparticles (SPIONs) and layered double hydroxides (LDHs) used in this thesis.

1.5.1. SPIONs in Biomedical Applications

Magnetic nanoparticles have become a field of intense development, widely explored in material chemistry, physics, biology and medicine [30]. A variety of synthetic methods to prepare magnetic nanoparticles of various compositions have been developed [93].

Among a number of magnetic nanoparticles of different various compositions, iron oxides have been widely investigated, for example as magnetic recording media, catalysts, and gas sensors [30]. Iron oxides exist in a range of structures and hydration states [30]. These materials have many attractive qualities, including low toxicity, good biocompatibility, and magnetic properties which enable them to be used as multifunctional vehicles for modern theranostics [28, 30].

1.5.1.1 Structures

Magnetite (Fe₃O₄) belongs to the ferromagnetic oxide (or ferrite) family. The iron cations in Fe₃O₄ are present in two valence states, Fe²⁺ and Fe³⁺ ([Fe³⁺]Td[Fe³⁺Fe²⁺]O₄). Magnetite crystallographically possesses a cubic inverse spinel form at 120K to 840K (Tc, the Curie temperature) [227]. The oxygen ions constitute a close-packed cubic lattice with the metal ions in interstices between them. Figure 1-16 shows two different interstitial holes where the iron ions can be located: tetrahedral (Td) sites and octahedral (Oh) sites. While the Oh sites are populated by both Fe²⁺ and Fe³⁺ ions, Td sites are by populated only with Fe³⁺ ions [227]. Under certain circumstance, magnetite (Fe₂O₄) can be transformed into maghemite (γ-Fe₂O₃, 0.75[Fe³⁺]Td[Fe³⁺V⁺¹/₃]OhO₄), which differs by the emergence of vacancies in the octahedral sites. These cationic vacancies can be ordered, partially ordered or totally random [99].
1.5.1.2 Synthesis and Stabilization of Magnetic Nanoparticles

The synthetic approach to magnetite nanoparticles usually includes the coprecipitation of a solution of a mixture of ferrous and ferric (Fe$^{2+}$/Fe$^{3+}$) salts with a base under an inert atmosphere [228]. This coprecipitation is a simple and efficient route which can be performed at large scale. However, control of particle size distribution remains a problem. A variety of approaches have been explored to tailor size, including the adjustment of pH, ionic strength, temperature, the nature of the salts used, or the ratio of Fe$^{2+}$/Fe$^{3+}$ [99]. Other methods to produce iron oxide nanoparticles of well-defined size include hydrothermal and high-temperature reactions, or sol-gel reactions [99].

To avoid the oxidation of magnetite nanoparticles and maintain the colloidal stability of the particles against aggregation, it is importance to stabilize the particles.

Figure 1-16 A schematic representation of the two different cation sites in magnetite.
1.5.1.3 Biomedical Applications

The pharmacokinetics and biodistribution of iron oxide nanoparticles are determined by their overall size. Phagocytic cells will rapidly take up large nanoparticles of > 200 nm, resulting in their accumulation in the monocyte phagocyte system (MPS), including in liver and spleen macrophages [96]. The kidneys will quickly eliminate small particles below 5.5 nm in size [229, 230]. SPIONs that avoid MPS uptake can persist for longer in the circulation (blood half-life ca. 2h), maximizing their chance to reach the targeted area [96].

Malignant tumours or metastases usually have a lack of Kupffer cells, a particular type of macrophage in the MPS. They thus differ from normal tissues in terms of nanoparticle uptake. In such cases, a distinct contrast between healthy tissue and tumours can be achieved in T₂-weighted images [90]. Due to the EPR effect, SPIONs with hydrodynamic diameter of < 100 nm can also passively invade small solid tumours and metastatic cells by crossing from the leaky vasculature into the interstitium [230].

Magnetic nanoparticles also have been applied in hyperthermia to treat tumours non-invasively via remote stimuli, such as applying an external magnetic field or near infrared (NIR) laser [231, 232].

1.5.2. Metal Hydroxide Nanocomposites

2D structured metal oxides and hydroxides have attracted extensive attention in the areas of electrochemistry, catalysts, electrochemistry, separation technology and medical science [233, 234]. Among these inorganic materials, layered double hydroxides (LDHs), stand out for biomedical applications because of their 2D structure, with high specific surface areas and ion exchange abilities [233, 234].

1.5.2.1 Structures

The structure of layered double hydroxides (LDHs) is related to the crystal structure of brucite, Mg(OH)₂, where the Mg²⁺ cation is octahedrally coordinated by 6 hydroxide ions (OH⁻). Each octahedral unit is linked to adjacent units by sharing edges to construct an infinite 2D sheet, resulting in a lamellar-structure. LDH sheets have similar structures but are positively charged due to the isomorphous replacement of Mg²⁺ cations by trivalent metals. The formula of LDHs is described as F[M(II)₁₋ₓ M(III)ₓ]
Simultaneously, interlayer anions solvated with H₂O are intercalated between the positively charged layers to ensure charge neutrality, resulting in a 3D layered structure, held together by Coulombic attraction between the layers and interlayer anions, as well as hydrogen-bonding interactions between hydroxide groups of the layers and electronegative atoms of the intercalated anions.

The structure of layered double hydroxides are presented in Figure 1-17. M(II) and M(III) refer to divalent and trivalent metal cations, respectively, and Aⁿ⁻ is interlayer anion. The general molar ratio (x) of M(III) to (M(II) + M(III)) suitable for the synthesis of an LDH is between 0.2 to 0.33. If x > 0.33, the high density of trivalent metal cations (M³⁺) will cause the precipitation of M(OH)₃ and similarly, at x < 0.2, the increasing number of M²⁺ ions will result in the formation of M(OH)₂ [235].

Figure 1-17 Schematic representation of the structure of an LDH (M(II) and M(III) refer to divalent and trivalent metal cations). Diagram reproduced from [234] with permission. Copyright © 2018 The Chemical Society of Japan & Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

1.5.2.2 Synthesis of LDH Nanocomposites

The synthetic approaches used to prepare LDH nanocomposites are illustrated in Figure 1-18 and Table 1-6. The most commonly used way is co-precipitation, also known as the direct method (Figure 1-18A). Typically, a solution containing divalent and trivalent metal cations is slowly mixed with a solution of guest anions to be intercalated, while
pH of the solution is kept constant (usually weakly basic) by slow addition of a base. To obtained well-crystallized nanocomposites, thermal treatment is often required [236].

![Diagram of synthetic routes to prepare LDH nanohybrids](image)

**Figure 1-18** The variety of synthetic routes which can be used to prepare LDH nanohybrids. (A) Coprecipitation, (B) ion-exchange, (C) reconstruction and (D) exfoliation-reassembling.

![Table 1-6](table)

**Table 1-6** The advantages and disadvantages of different approach to synthesize LDHs [234, 236]

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-precipitation</td>
<td>High yields, facile</td>
<td>Thermal post-treatment may be needed, limited-size anions can be intercalated</td>
</tr>
<tr>
<td>Reconstruction method</td>
<td>Ability to intercalate organic molecules</td>
<td>Low yields, poor crystallization, partial intercalation</td>
</tr>
<tr>
<td>Anion-exchange</td>
<td>Large-sized anions can be incorporated</td>
<td>Low yields</td>
</tr>
<tr>
<td>Exfoliation-reassembling</td>
<td>Ability to intercalate bulky and large-sized biomolecules</td>
<td>Low yields</td>
</tr>
</tbody>
</table>

The conventional aqueous co-precipitation system also can be introduced into a reverse micelle, where monolayer LDHs can be produced due to the restricted space and materials available for particle growth inside the reverse micelle. Such micellar systems
usually comprise an oil phase with surfactant (e.g. DDS) and cosolvent (1-butanol) [237, 238].

Another common synthetic route is anion exchange (or indirect method, Figure 1-18B). Using this method, various anions have been intercalated between the host layers [233, 234]. In the first stage, an LDH precursor is formed by the co-precipitation method [239]. Next, the initial interlamellar anions are replaced by the desired anions by stirring the precursors in a solution with an excess of the replacement guest anions. In some circumstances, the whole process should be performed under an inert atmosphere to prevent the intercalation of CO$_3^{2-}$ [240].

The reconstruction approach is also a well-established synthetic route (Figure 1-18C). This approach is dependent on a property of LDHs: the process of calcination-rehydration can induce their reconstruction to the original structure. Generally, a mild calcination process at temperature around 400 to 500 °C completely removes the hydroxyl groups of LDH and the intercalated anions, and the LDH is transformed to a mixed metal oxide [241, 242]. These can reconstruct to their original LDH structure when reacted with water or a solution of anions (rehydration) [239]. This method is particularly helpful for the intercalation of organic anions, but it should be noted that rehydration should be conducted in the presence of an inert atmosphere to avoid contaminations.

Exfoliation-reassembling (Figure 1-17D) is also a good method for intercalation of a range of different anions, such as polymers, DNA [234, 237]. This method involves: 1) synthesis of LDH monolayer sheets, 2): reassembly of the sheets with guest anions. Bottom up route was also reported [243]. By adding a high concentration of formamide to the traditional aqueous coprecipitation, single layer nanosheets can be obtained in a single step. This is because the formamide strongly interacts with the charged LDH sheets, significantly inhibiting further growth of the layer [243].

1.5.2.3 Biomedical Application

LDHs have been widely explored in biomedical applications. Hydrotalcite, a classic MgAl-LDH with the formula [Mg$_6$Al$_2$(CO$_3$)(OH)$_{16}$·4H$_2$O] has been commercialized by Bayer, with the product name of “Talcid®” [244]. It is prescribed for neutralizing the gastric acid of patients with acute or chronic gastritis. A wide range of active ingredients
can be intercalated into the interlayer spaces of LDHs, e.g. small drug [245, 246], antibiotics [234], DNA [247, 248], vitamin C [249, 250].

Active agents intercalated into LDHs can be released in a controlled manner at the target area. This capacity for localised sustained release can protect the agents from degradation, alleviate undesirable release outside the target site, and therefore minimise side effects, avoid toxicity and reduce dosing concentration and frequency, thereby improving the overall bioactivity [235, 251].

Another characteristic of LDH nanocomposite delivery systems is their pH sensitive properties. This is related to the nature of metal hydroxides. A number of drug release experiments have revealed that in acidic conditions (e.g. pH 4.8, which mimics the environment in lysosomal conditions), the release of a drug from LDH-nanocomposites is faster than at the physiological pH (7.4) [251, 252].

1.6. Pharmaceutical Engineering

Medication formulating is a key point in pharmaceutical engineering. Formulations are developed for all the main routes of administration, and a variety of pharmaceutical fabrication techniques have been applied for advanced drug delivery [253-255]. Here we will focus on two of them, electrohydrodynamic atomization (EHDA) and spray drying. Both of them possess unique advantages and were used in this thesis.

1.6.1. Electrohydrodynamic Atomization (EHDA) Techniques

1.6.1.1 Electrospinning/Electrospray Apparatus

EHDA is a versatile and facile technique that is widely explored in various fields including tissue engineering, biosensors, wound dressings and drug delivery [256, 257]. In the process, fine jet or droplets are generated from the atomization of a liquid under electrical forces [258]. EHDA encompasses electrospinning (to fabricate fibres) and electrospraying (to generate particles). Via the application of a high voltage to a polymer solution, repulsion of electrical charges building in the solution results in a conical shape (known as the Taylor cone), from which a thin liquid jet (electrospinning) or tiny droplets (electrospray) is ejected when the electrostatic force overcomes the surface tension. This jet or droplets subsequently undergo Coulombic explosion and was split
into smaller droplets, and travels towards a collector, resulting in fibres or particles, respectively (Figure 1-19) [257].

Figure 1-19 A schematic representation of the laboratory setup for single-fluid EDHA. The inset is a sketch of the electrified Taylor cone.

Besides conventional EHDA (single-fluid, Figure 1-19) employing a polymer solution, co-axial EDHA is also a facile technique to fabricate nanofibres or particles containing multiple compartments [259]. As shown in Figure 1-20 [259], two separate solutions are employed for injection in a co-axial setup.

Figure 1-20 A schematic representation of the laboratory setup for co-axial fluid EDHA.
Chapter 1

1.6.1.2 Pharmaceutical applications

EHDA is an attractive technology that has been successfully applied in pharmaceutical science to produce micro- and nanoscale structures with a mono-dispersed size [260]. Its advantages involve [260-263]:

- EHDA enables delivery platforms for a variety of active agents, e.g. small molecule, nanoparticles, peptide, proteins.
- Micron and nanoscale structures are engineered in a controlled fashion (constant rate, no heating) with the rapid evaporation of solvent.
- A number of structure types e.g. particles, fibres, bubbles and printed architectures.
- Structures with various phases (solids, liquids and gases) can be generated.

**Microparticles**

Micro- and nanostructure formulations of hydrophobic drugs can be simply prepared via EHDA by dissolving the drug and a polymer(s) in an organic solvent [264, 265]. Hydrophilic compounds are usually dissolved in an aqueous solution and then blended with a polymer / organic solvent solution to give an emulsion for EDHA [266].

Besides encapsulation of a single drug, microspheres within hydrophilic and hydrophobic drugs in the core and shell or vice versa can be prepared using co-axial EHDA and have been reported for the concurrent and sequential release of multiple drugs [253, 254]. This is particularly promising in cancer therapy and the treatment of diseases where ‘cocktail-therapy’ is required [253, 254]. Besides, the experimental conditions required for EHDA are mild and flexible, which makes it an intriguing approach to fabricate uniform sized and biologically active nanoparticles [267].

EHDA has also been applied in the fabrication of pH-sensitive and targeted delivery systems. Encapsulation of doxorubicin in elastin polypeptide microspheres by EHDA was found to result in acid responsive release of the drug [268]. In other work, an acid-sensitive core-shell nanoparticle was prepared using co-axial electrospraying and subsequently linked with an antibody for the purpose of tumour-targeted, pH responsive drug delivery [269]. The results of in vitro drug release and cytotoxicity tests showed
the formulation’s targeting potential in cancer treatment [269]. EHDA has also been applied in the encapsulation of MRI contrast agents. For instance, SPIONs within PLGA microspheres have been produced by co-axial electrospaying, and showed potential application as T₂ contrast agents [270]. Therefore, particles fabricated by electrospaying can be utilized simultaneously for both diagnostic and therapeutic applications. To this end, Si et al. used co-axial electrospaying to load both imaging agents and a drug into PLGA microparticles [271].

**Fibres**

Electrospun fibres also have attracted increasing attention for their capacity to easily incorporate a wide range of functional components such as small molecules, inorganic nanoparticles or proteins [272, 273].

For example, Zeng et. al prepared electrospun poly(L-lactic) acid (PLLA) nanofibres loading hydrophilic cytotoxic agent, paclitaxel as anti-cancer therapy [274]. Another electrospun fibres incorporates hydrophobic ibuprofen (an anti-inflammatory drug) into hydrophilic matrix polyvinylpyrrolidone (PVP) for oral fast-dissolving delivery [275]. Eudragit fibres also have been prepared via electrospinning as delivery systems. Jin et al. fabricated core/shell fibre systems for simultaneous MR Imaging and drug delivery for colonic cancer [276]. This platform was easily prepared using co-axial electrospinning, and comprises a pH-responsive Eudragit shell and poly(ethylene oxide) cores loaded with indomethacin and Gd(DTPA). The pH-responsive shell allows the active agents to be co-delivered to the colon, where PEO core will adhere to the walls of the intestine and swell to sustainably release active ingredients.

**1.6.2. Spray Drying**

Spray drying is also a well-established technique used in pharmaceutical fabrication, which involves formulation of a solutions, suspensions or emulsion via solvent evaporation [277].
1.6.2.1 Apparatus

Typical spray drying apparatus is shown in Figure 1-21. A mixture of polymer matrix and other agents (as solution, emulsion or dispersion) is converted into droplets by an atomiser. These droplets then enter the drying chamber where, at a controlled temperature, hot air flowing in a constant rate leads to the rapid evaporation of solvent. The resulting dried particles fall through the gas medium into the collector.

![Figure 1-21](image_url)  
Figure 1-21 A schematic diagram showing spray drying apparatus. Taken from [277] with permission. Copyright © 2007 Elsevier Ltd. All rights reserved.

1.6.2.2 Applications

Spray drying has been widely used in a number of areas, including food, ceramics, biomedical industries [278, 279]. Spray drying has been reported as an encapsulation method to produce controlled and sustained release formulations. Esposito et al. used this route to fabricate microcapsules encapsulating vitamin C in the pH-responsive polymers Eudragit RL, RS, and L, aiming to achieve colon-targeted delivery [278]. Takahashi et al. use a natural pH-responsive polymer, chitosan, to encapsulate paracetamol by spray drying. The resulting microparticles displayed markedly delayed release, since the paracetamol was bound to chitosan through hydrogen interactions [279]. It also have been used to develop anticancer microspheres for controlled release [280, 281]. For example, poly (D,L-lactide) microparticles with chemotherapy could obtain long-acting release by appropriate operating parameters of spray drying [280].
A comparison of EHDA and spray drying is given in Table 1-7 [282]. In this work, both techniques will be explored.

Table 1-7 Comparison between spray drying and EHD. Adapted from [282]

<table>
<thead>
<tr>
<th>Properties</th>
<th>EHDA</th>
<th>Spray drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cost-effective</td>
<td>High</td>
<td>High, but required large first capital investment</td>
</tr>
<tr>
<td>Scalability</td>
<td>Moderate</td>
<td>Easy</td>
</tr>
<tr>
<td>Use of heat</td>
<td>No heat involved</td>
<td>Use of heat, thus degradation of thermosensitive materials</td>
</tr>
<tr>
<td>Adaptable to product specifications</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Control over physicochemical parameters</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>One step process</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Yields</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Steps</td>
<td>Single-step</td>
<td>Single-step</td>
</tr>
<tr>
<td>Continuity</td>
<td>Continuous</td>
<td>Continuous</td>
</tr>
<tr>
<td>Speed</td>
<td>Medium-Low</td>
<td>High</td>
</tr>
<tr>
<td>Versatility</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Final drying step</td>
<td>Not required</td>
<td>Not required</td>
</tr>
<tr>
<td>Effective encapsulation of drugs within polymer carriers</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.7. Project Hypothesis

Recent advances in bioimaging, biochemistry and bioinformatics have facilitated the development of personalized and precise medicine. Theranostics, as a representative of next-generation medicine combined imaging modalities and therapeutic approach, have garnered a lot of attention due to the potential to achieve a visible and controllable treatment for individual patients. A promising strategy to achieve this potential involves the development of therapy guided by MRI with high degree of soft tissue contrast, low invasiveness, depth of penetration and spatial resolution. With the precise and timely assessment of disease condition and therapeutic outcome, MRI-guided therapy can become highly efficient and specific for treatment and diagnosis.

This work focuses on developing advanced MRI-guided theranostics and the aims are as follows:

- Carmofur and SPIONs will be used as the model drug and MRI contrast agent, respectively to optimise the EHDA processing parameters and produce a series of pH-responsive oral formulations for MRI-guided chemotherapy.
- Co-axial EHDA will be employed to develop dual-responsive systems with distinct structures. PNIPAM and Eudragit will be examined as thermo and pH-responsive component, and the formulations are designed for oral delivery of carmofur and SPIONs to small and intestinal areas.
- Thermo-responsive microparticles will be formulated via spray drying. These formulations exploiting nano-in-micro strategy will carry chemotherapeutics agents (MTX or 5FU) and SPIONs, $T_2$ MRI contrast agents and will be explored as MRI-guided platforms for hyperthermia-enhanced chemotherapy.
- Gd doped LDHs will be fabricated and optimised to have good colloidal stabilities and high relaxivity. Then the nanosheets will be loaded with MTX or 5FU as theranostic systems for MRI-guided chemotherapy.
- A series of co-shell fibres will be developed via EHDA using polycaprolactone or poly(D,L-lactide-co-glycolic). The fibre materials will be further functionalized via PDA modifications to obtain implantable theranostic platform for anticancer therapy. Their ability to locally and substantially deliver MTX, as well as their MRI contrast ability will be investigated.
1.8. References

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Chapter 2

Methods
2.1. Introduction

This chapter will describe the general techniques and methods employed for all the formulations reported in this thesis. Materials, experimental details and syntheses specific to a particular chapter will be detailed in full in the corresponding chapter.

2.2. Nanoparticle Preparation

2.2.1. PVP-stabilized Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

2.2.1.1. Materials

Chemicals were purchased as follows: sodium hydroxide and hydrous ethanol (Fisher Scientific); acetone, anhydrous ethanol, polyvinylpyrrolidone (PVP; 40 kDa), FeCl₃·6H₂O and FeCl₂·4H₂O. Ultrapure water was collected from a Millipore MilliQ system operated at 18.2 MΩ.

2.2.1.2. SPIONs Synthesis

FeCl₃·6H₂O (6.5 g, 0.024 mol) and FeCl₂·4H₂O (2.48 g, 0.012 mol) were dissolved in 25 mL of deoxygenated deionized (DI) water. This solution was added dropwise into 250 mL of an aqueous NaOH solution (0.5 M) at 40 °C, and stirred for 1 h at this temperature. The resultant SPIONs were washed by centrifugation with DI water until the supernatant was pH neutral, and the resultant black precipitate dried under vacuum.

2.2.1.3. PVP-stabilized SPIONs

The stabilization method is based on [1]. SPIONs (100 mL, 10 mg/mL in water) were mixed with 2 mL of an aqueous PVP 40 kDa solution (25.6 g/L, 0.64 mM), and the suspension was shaken (100 rpm) at room temperature. After 24 h, the suspension was mixed with aqueous acetone (H₂O/acetone, 1:10 v/v) and centrifuged at 13,200 rpm for 20 min. The supernatant was removed, and the resultant black precipitate washed with ethanol and dried in an oven at 50 °C for 24 h.
2.2.2. Drug Loaded Layered Double Hydroxides (LDH)

2.2.2.4. Materials

Sodium hydroxide was purchased from Fisher Scientific Ltd. AlCl₃·6H₂O, MgCl₂·6H₂O and 5-fluorouracil (5FU) were sourced from Sigma-Aldrich. Methotrexate (MTX) was obtained from Generon.

2.2.2.5. Synthesis of Mg₃Al-LDH

The reaction was based on a literature protocol [2]. In a typical procedure, a salt solution (10 mL) containing MgCl₂·6H₂O (0.6 M, 1.22 g) and AlCl₃·6H₂O (0.2 M, 0.48 g) was quickly added into an aqueous NaOH solution (40 mL, 0.4 M, 0.64 g) while stirring vigorously. After stirring for 20 min, the fresh Mg₃Al-LDH slurry was collected via centrifugation (9000 rpm, 20 min), washed twice with 20 ml of deionized water, and re-suspended in deionized water (40 mL). Next, the slurry was heated at 100 °C for 6 hours in a 50 mL Telfon-lined stainless steel autoclave (Figure 2-1), followed by product recovery through centrifugation and washing with deionized water until the supernatant pH was close to 7. The resultant LDH was dried under vacuum.

![Figure 2-1 Schematic illustration (A) and photograph (B) of the Telfon-lined stainless steel autoclave.](image)

2.2.2.6. Drug Loading

400 mg of the LDH was dispersed in an aqueous solution (20 mL) containing MTX (0.06 mmol, 455 mg) or 5FU (0.1 mmol, 390 mg) at pH 9.5. After stirring at 40 °C for 48 hours, the product was washed with deionized water through centrifugation. After a
final wash with acetone, the MTX or 5FU loaded Mg$_3$Al-LDH (LDH-MTX, LDH-5FU) was dried at 40 °C for 48 hours in an oven.

### 2.3. Electrohydrodynamic Atomization (EHDA)

#### 2.3.1.1. Single Fluid Process

An HCP 35-35000 power supply (FuG Elektronik GmbH) was used to generate an electric field. A 5 mL plastic syringe (Terumo, UK) fitted with a narrow-bore stainless-steel needle (18G, with outer and inner diameter of 1.25 and 0.838 mm) was filled with the required working solution. The spinneret was connected to the positive electrode of the power supply via an alligator clip and a flat plate aluminium collector attached to the grounded electrode. The working solution was dispensed with the aid of a syringe pump (KDS 100, Cole-Parmer) under ambient conditions (22 ± 3 °C and relative humidity 40 ± 5%).

#### 2.3.1.2. Co-axial Fluid Process

The co-axial EHDA set up is given in Figure 2-2. This is very similar to the single fluid process, except that two syringe pumps (KDS 100, Cole-Parmer) were employed to control the sheath and the core flow rates.

![Figure 2-2 Photograph of the co-axial EHDA set-up.](image)
Chapter 2

Methods

2.4. Characterization

2.4.1. Morphological Characterization

2.4.1.1. Scanning Electron Microscopy (SEM)

A field emission scanning electron microscope (FEI Quanta 200F) connected to a secondary electron detector (Everheart-Thornley Detector-ETD) was employed. Samples were coated with a 20 nm gold-sputter (Quorum Q150T) prior to imaging. The size distribution was determined from the SEM micrographs using the ImageJ software (version 1.52s, National Institutes of Health) to measure the diameter of at least 100 fibres or particles.

2.4.1.2. Transmission Electron Microscopy (TEM)

For magnetic samples, TEM images were obtained on a JEOL JEM-1200 microscope operated at 120 kV with a beam current of ca. 80 mA. A Gatan Orius 11 megapixel camera was used to take images.

For samples without magnetic components, Transmission electron microscopy images of the specimens were taken using a Philips CM 120 Bio-Twin instrument (Philips/FEI Corporation, Eindhoven, Netherlands), which were conducted with the help of Dr Andrew Weston (School of Pharmacy, UCL). Size distributions were determined using ImageJ software (version 1.52s, National Institutes of Health) and measuring the diameter of at least 100 objects.

2.4.1.3. Digital Microscopy

Transmitted light images were obtained from an EVOS XL Cell Imaging System digital inverted microscope (ThermoFisher Scientific, UK).
2.4.2. Physiochemical Characterization

2.4.2.1. Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared (FTIR) analyses in Chapter 3 to 6 were carried out on a Spectrum 100 instrument (PerkinElmer Co. Ltd, Waltham, Massachusetts, USA) over the range 650-4000 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\).

In Chapter 7, FTIR analysis were carried out in attenuated total reflectance mode on an Eco-ATR IAlpha spectrometer (Bruker), over the range 450-4000 cm\(^{-1}\).

2.4.2.2. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis was undertaken on a Discovery TGA (TA Instruments, Waters LLC). Ca. 3 mg of each sample was loaded into an aluminium pan and heated from 40 to 500 °C at 10 °C/min under a nitrogen flow of 25 mL/min. Data were recorded using the Trios software and analysed with TA Universal Analysis.

2.4.2.3. Differential Scanning Calorimetry (DSC)

A Q2000 DSC (TA Instruments, Waters LLC) was used to analyse samples. A small amount of sample (approximately 3 mg) was loaded in a non-hermetically sealed aluminium pan (T130425, TA instruments) and DSC experiments carried out from 40 to 126 °C, with a temperature ramp of 10 °C/min and nitrogen purge of 25 mL/min. Modulated temperature DSC (MDSC) were conducted from 0 to 200 °C with a temperature ramp of 3 °C/min (modulate +/- 1 °C). DSC data were recorded with the TA Advantage software package and analysed using TA Universal Analysis.

2.4.2.4. X-ray Diffraction (XRD)

A MiniFlex 600 diffractometer (Rigaku) supplied with Cu-K\(\alpha\) radiation was used to collect XRD patterns (\(\lambda = 0.15418\) nm, 40 kV, 15 mA). Patterns were recorded over the 20 range from 3 to 70° (step = 0.01°).

2.4.2.5. Nuclear Magnetic Resonance (NMR)

NMR experiments were performed on a Bruker Avance 400 MHz spectrometer, with DMSO-d6 as the solvent.
2.4.2.6, Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES)

Samples were digested using a few drops of concentrated HNO₃, and then diluted to 10 mL with DI water. The iron or gadolinium concentrations (mM) were quantified on an Agilent 7500cx spectrometer. ICP-OES measurements were carried out by Connor J. R. Wells (Department of Chemistry, UCL).

2.4.2.7, Energy-Dispersive X-ray

Energy dispersive X-ray spectroscopy (EDX) was carried out using a Carl Zeiss EVO 25 SEM with an Oxford Instruments X-max 80 EDS detector (work in Chapter 5) or a Hitachi S3400N scanning electron microscope (work in Chapter 7). The accelerating voltage was 20 kV, with a working distance of 8.5 mm. The Oxford Instruments Aztec 5.0 software was used to collect EDX data.

2.4.3, Additional Characterization Techniques

2.4.3.1, Dynamic Light Scattering (DLS)

2 mg of sample was dispersed into 2 mL of phosphate-buffered saline (PBS) solution, deionized water or ethanol. After sonication for 30 min, samples were measured on a Zetasizer Nano ZS instrument (Malvern Instruments). A 4 mW He-Ne 633 nm laser module was employed and scattered light was measured at 173° (back scattering). The attenuator and position was selected automatically by the instrument and particle sizes are reported as the average of 5 measurements.

Zeta potential measurements were performed at 25 °C. Samples were loaded into a DTS1070 disposable folded capillary polystyrene cell (Malvern Instruments Ltd.). The cell was pre-washed with absolute ethanol and dried prior to use. The zeta potential was calculated with the Zetasizer software version 7.11 (Malvern Instruments Ltd.), using Smoluchowski’s equation with a Henry’s function (f(Kα)) of 1.50.

2.4.3.2, Proton Relaxivity

An MQC+ benchtop NMR analyser (Oxford Instruments) was used to measure longitudinal (T₁) and transverse relaxation times (T₂) of protons at 37 °C and 23 MHz. The standard inversion-recovery method was employed to determine T₁, with a typical
90° pulse calibration of 250 μs and 4 scans per experiment. The Carr-Purcell-Meiboom-Gill (CPMG) method was used to measure $T_2$, with 4 scans per experiment. The water relaxation rate enhancement per mmol of contrast agent is defined by Equation 2-1.

$$r_{1,2} = \frac{R_{1,2,\text{obs}} - R_{1,2,\text{sol}}}{[\text{CA}]}$$  \hspace{1cm} \text{Equation 2-1}$$

Where $R_{1,2,\text{obs}}$ is the observed relaxation rate of the agent in aqueous suspension ($R_{1,2} = 1/T_{1,2}$), $R_{1,2,\text{sol}}$ is the relaxation rate of the unaltered solvent system (i.e. in the absence of contrast agent) and [CA] is the mM concentration of the contrast agent in suspension, as measured by ICP-MS.

### 2.4.3.3. Microplate Reader

A SpectraMax M2e microplate reader (Molecular Devices, UK) was utilized to read absorbance and fluorescence. The instrument was equipped with dual monochromators and a high-power Xenon flash lamp. The reader was controlled by the SoftMax Pro software, version 6.3 (Molecular Devices, UK).

### 2.5. Drug Loading and Encapsulation Efficiency

The loading capacity (LC %) can be calculated as the amount of entrapped drug divided by the total formulation weight. Encapsulation efficiency (EE %) is the percentage of the drug present that is successfully entrapped into the formulation.

#### 2.5.1. Carmofur Loaded Formulations

The following protocol was used for the carmofur loaded formulations described in Chapter 3 and 4. 10 mg of the composites (n=5) was added into 10 mL of ethanol and sonicated until the polymer was fully dissolved. A PVDF-type syringe filter (0.22 μm) was used to filter the resultant solutions, and the filtrates centrifuged for 10 min (13,200 rpm) to remove the SPIONs. The supernatants were analyzed with UV spectroscopy at 262 nm (Cary 100 instrument, Agilent), and the LC % and EE % calculated based on a pre-determined calibration curve.
2.5.2. Drug-Loaded Layered Double Hydroxides (LDHs)

The following protocol was used for the methotrexate (MTX) or 5-fluorouracil (5FU) loaded LDHs or Gd-doped LDHs produced in Chapter 5-7. 2 mg of drug loaded LDH or Gd-doped LDH powder was charged in a 10 mL volumetric flask and suspended in 5 mL of DI water. The solution was acidified with one droplet of concentrated HCl, and shaken until the LDH was fully degraded. After neutralizing with 0.2 M aqueous NaOH, the solutions were made up to 10 mL with DI water. The resultant solution was filtered through a 0.22 μm PVDF-type syringe filter before being analysed with UV spectroscopy at 262 nm for 5FU and 303 nm for MTX (Cary 100 instrument, Agilent).

2.5.3. Formulations Containing Drug LDHs

2.5.3.1. Drug Loaded Thermo-Responsive Microparticles

For the drug loaded microparticles prepared for Chapter 5, 2 mg of the microparticles (n=5) was placed in a 10 mL volumetric flask and suspended in 5 mL of DI water. The resultant suspensions were heated at 50 °C until the polymer was fully dissolved. 1-2 droplets of concentrated HCl were added to fully degrade the LDH nanocomposites, and the pH then adjusted to around 7 using 0.2 M aqueous NaOH. Finally, the volume of each solution was made up to 10 mL with DI water. A PVDF-type syringe filter (0.22 μm) was used to filter the resultant solutions before they were analysed with UV spectroscopy as above.

2.5.3.2. Polydopamine (PDA) Coated Fibres

The following protocol was used for polydopamine (PDA) coated fibres described in Chapter 7. The amount of MTX loaded into fibres was determined as follows. Around 5 mg of fibres was added into 20 mL of dichloromethane (DCM) and sonicated until the fibres fully dissolved. The resulting suspensions were centrifuged for 5 min (8,000 rpm) to remove undissolved particles. Next, the sediment was dried under vacuum to remove any remaining DCM, and 10 mL of a 1 w/v % sodium hypochlorite aqueous solution added to completely dissolve any remaining particles. 5 mL samples of the solutions were removed and diluted to 50 mL in a volumetric flask. The MTX loading was calculated based on the concentration of MTX in the supernatant and sediment, both
determined by high performance liquid chromatography (HPLC). Quantification was performed in triplicate.

2.6. Analysis of Drug Release Data

2.6.1. Kinetic Models

The release profiles were mathematically modelled using a series of models, as detailed below.

2.6.1.1. Korsmeyer Peppas Model

Korsmeyer and Peppas devised a semi-empirical model to specifically determine the release mechanism of a drug molecule from polymeric matrices, e.g., a hydrogel, as given in Equation 2-2 [3]. The equation considers two apparently independent phenomena of drug release, relaxation, and diffusion.

\[
\frac{M_t}{M_\infty} = k t^n \quad \text{Equation 2-2}
\]

\(M_t/M_\infty\) represents the extent of drug release (less than 0.6 for the equation to be applied), \(k\) is a rate constant, and \(n\) gives information related to the mechanism of release. In all circumstances, the Korsmeyer Peppas model assumes that the release takes place only in one direction and that the ratio between the width and thickness of the systems is no more than 1:10. Table 2-1 outlines the classification of release behaviour from polymeric matrix systems with different geometries.
Table 2-1 Classification of release behaviours of active agent from polymeric matrix systems with different geometries

<table>
<thead>
<tr>
<th>Release model</th>
<th>Geometry</th>
<th>Release exponent ((n))</th>
<th>Release mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fickian diffusion</td>
<td>Planar (thin films)</td>
<td>0.50</td>
<td>Diffusion controlled. The release kinetics are characterized by the solvent transport rate (diffusivity).</td>
</tr>
<tr>
<td></td>
<td>Cylinders</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spheres</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>Anomalous transport</td>
<td>Planar (thin films)</td>
<td>0.50 to 1.0</td>
<td>Diffusion and swelling/relaxation of polymeric chains controlled. The swelling of polymeric chains takes place slowly and the diffusion process simultaneously result, in time-dependent anomalous conditions.</td>
</tr>
<tr>
<td></td>
<td>Cylinders</td>
<td>0.45 to 0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spheres</td>
<td>0.43 to 0.85</td>
<td></td>
</tr>
<tr>
<td>Case II transport</td>
<td>Planar (thin films)</td>
<td>1.0</td>
<td>Swelling or relaxation of polymeric chains controlled. The process of relaxation on the gel-vitreous polymeric interface limits the solvent penetration and diffusion, therefore determining the release kinetics.</td>
</tr>
<tr>
<td></td>
<td>Cylinders</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spheres</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Super Case II transport</td>
<td>Planar (thin films)</td>
<td>&gt; 1.0</td>
<td>During the sorption process, tension arising from the gel outer layer produces compression on the nucleus. As the polymeric interfaces move to the nucleus, the tension continually rises until the nucleus collapses.</td>
</tr>
<tr>
<td></td>
<td>Cylinders</td>
<td>&gt; 0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spheres</td>
<td>&gt; 0.85</td>
<td></td>
</tr>
</tbody>
</table>

2.6.1.2. Zero-order Model

Zero-order release describes concentration independent release \((Equation 2-3)\) [4].

\[
\frac{Q_t}{Q_0} = k_0 t
\]

\(Q_0\) is the initial loading amount, \(Q_t\) refers to the cumulative amount released at time \(t\), and \(k_0\)the is the rate constant. Zero-order release can be achieved using a number of drug delivery systems such as transdermal slow release matrices, coated, and osmotic platforms. For example, coating a core tablet can realize zero-order release via oral route [4]. If both water and drug in the core can permeate the coating matrix, the core will be hydrated after oral admiration. As a result, the drug dissolves until reaching its saturation or solubility. Subsequently, the drug in the saturated reservoir is freed by partition from the reservoir into the coating matrix, diffusing through the swelling matrix, finally being free into the gastrointestinal fluids. The constant release rate is dependent on the drug
saturation in the core, leading to a stationary concentration gradient across the coating matrix [5]. If the drug concentration decreases below saturation, the release velocity will decay to zero. Therapeutic systems where active agent can be released at a constant rate are desirable for sustained drug release to maintain effective drug concentrations \textit{in vivo} while avoid frequent repetitive dosing [6].

2.6.1.3. Bhaskar Model

The Bhaskar model (Equation 2-4) assumes that diffusion through a particle is the rate limiting step to release, making it appropriate for ion-exchange processes [7]. It can be used for evaluation of particle diffusion-controlled release of drug from drug-resin complex.

\[
\ln \left(1 - \frac{M_t}{M_{\text{inf}}} \right) = kt^{0.65} \quad \text{Equation 2-4}
\]

\(M_t\) is the amount of drug released at time \(t\), \(M_{\text{inf}}\) the total amount of drug loaded in the carrier, and \(k\) is a rate constant.

2.6.1.4. Avrami-Erofe'ev Model

The Avrami-Erofe'ev model (Equation 2-5) [8] was used to study how solids transform from one phase to another at constant temperature. It can illustrate a variety of kinetic process, including crystallisation, chemical reaction, drug release or even ecological system changes.

\[
\alpha = 1 - e^{(-kt^n)} \quad \text{Equation 2-5}
\]

Here, \(\alpha\) represents the fraction reacted till certain time, for drug release profile, \(\alpha = M_t/M_{\text{inf}}\), \(n\) is an exponent (Avrami constant) that provides information on the reaction mechanism, and \(k\) is a rate constant. The Avrami constant \(n\) is usually held to have a value between 1 and 4, which provides information for the nature of the transformation in question. For instance, value of 1 can be regarded to result from a constant nucleation rate, while 4 represent contributions from three dimensions of growth [9]. \(n\) possesses a value of 3 when the transformation is merely lead by the 3-dimensional growth of the nuclei, usually from the beginning of all the transformation present if nuclei are pre-formed. If nucleation takes place on specific sites (i.e., grain boundaries) that quickly saturate after the transformation starts, the situations are more complicated. Originally,
nucleation may be randomly occurred and growth unlimited, resulting in high values for Avrami constant (3 to 4). Once the nucleation places are exhausted, the formation of new particles will terminate. When the arrangement of nucleation sites is non-random, the growth may be restricted to 1 or 2 dimensions. Values of 1, 2 or 3 for Avrami constant can be due to site saturation for surface, edge and point sites, respectively [10].

2.6.2. Release Data Comparison

Two fit factors $F_1$ (the difference factor) and $F_2$ (the similarity factor) (Equations 2-6 and 2-7) were applied to statistically compare two dissolution profiles [11]. $R_t$ and $T_t$ represent the percentage of active pharmaceutical ingredient released from the reference and test samples at the time point $t$, respectively, and $n$ is the number of time points.

The difference factor ($F_1$) determines the percent error between two dissolution curves over total number of test points, and takes a value between 0 to 100. It is 0 if two curves are identical and tends to 100 when dissimilarity arises. The similarity factor ($F_2$) measures the logarithmic transformation of the sum-squared error of differences between the test dissolution curve and reference dissolution curve over total test points.

Two dissolution curves considered to be similar where the values of $F_2$ are close to 100. The decision guide lines for two dissolution profiles to be regarded as “the same” or “equivalent” is when $F_1$ is less than 15 and $F_2$ is greater than 50 [12]. Here we use the experimental release data obtained by UV-vis spectroscopy as the reference ($R_t$), and the predicted data as the test ($T_t$).

\[
F_1 = 100 \left( \frac{\sum|R_t-T_t|}{\sum R_t} \right) \quad \text{Equation 2-6}
\]

\[
F_2 = 100 - 25 \log \left[ 1 + \frac{\sum (R_t-T_t)^2}{n} \right] \quad \text{Equation 2-7}
\]

2.7. Cell Studies

2.7.1. Cell Culture

Dulbecco’s modified Eagle medium with high glucose, and heat-inactivated fetal bovine serum were purchased from Gibco (UK). Penicillin-streptomycin (1 % v/v), L-
glutamine (1 % v/v) solutions and non-essential amino acid solution (1 % v/v) were obtained from Life Technologies (UK).

The colorectal adenocarcinoma cell line Caco-2 (ATCC HTB-37) and A549 cells (ATCC CCL-18), an adenocarcinomic human alveolar basal epithelial cell line, were both employed for *in vitro* studies. Caco-2 cells were maintained at 37 °C, under 5 % CO₂, in Dulbecco’s modified Eagle medium with high glucose (DMEM-HG) supplemented with penicillin-streptomycin (1 % v/v), L-glutamine (1 % v/v), non-essential amino acid (1 % v/v) solutions and 10 % v/v heat-inactivated fetal bovine serum. A549 cells were cultured in DMEM-HG supplemented with pre-heated fetal bovine serum (10 % v/v), L-glutamine (1% v/v) and penicillin (1% v/v) solutions.

### 2.7.2. *In vitro* Cell Viability

#### 2.7.2.1. PrestoBlue™ Cell Viability Assay

For PrestoBlue assays, after the desired cell culture time and treatment, the wells were aspirated and cultures were washed with PBS, followed by addition of 20 μL of the fluorescent reagent and 180 μL of DMEM-HG medium for 96 well plates. After addition, the plate was incubated for 20 minutes at 37 °C and 5 % CO₂ before fluorescence (excitation/emission) at 560/590 nm was read using a SpectraMax M2e spectrophotometer (Molecular Devices, UK). The viability of the cells was calculated using Equation 2-8:

\[
Viability = \frac{\text{Fluorescence of cells exposed to formulations} - \text{background}}{\text{Fluorescence of untreated cells} - \text{background}}
\]

Equation 2-8

Cell viability data for different treatments were compared by Student's t-tests.

#### 2.7.2.2. Crystal Violet Staining

Crystal violet ([Figure 2-3](#)) is a triarylmethane dye that binds to ribose type molecules such as the DNA in nuclei.
Cell cultures were washed with PBS, fixed with 10% formalin for 15 min, and then incubated with 0.1 w/v % crystal violet solution for 20 min. After staining, cells were carefully washed three times with PBS and imaged on an Evos XL Core light microscope (ThermoFisher Scientific, UK).

2.8. References

Chapter 3

pH-Responsive nanocomposite delivery systems
3.1 Introduction

3.1.1 Superparamagnetic Iron Oxide Nanoparticles for Theranostics

Superparamagnetic iron oxide nanoparticles (SPIONs) are very good candidates for theranostics because of their unique physical and chemical properties [1]. By creating local magnetic field gradients, SPIONs can significantly decrease the transverse relaxation time ($T_2$) in magnetic resonance imaging (MRI) [2]. Although Gd-chelates are considered to be safe contrast agents, it has been reported in some rare cases that people with renal disease may suffer severe medical conditions referred to as ‘nephrogenic systemic fibrosis’ after administration of Gd-chelates. SPIONs are safer and can be metabolized by normal biochemical pathways. The magnetic behaviour of SPIONs also lends them to other biological applications [3, 4]. By applying an external magnetic field, SPIONs can be targeted to a desired location and induced to generate localised heating [5, 6]. These properties allow SPIONs to be employed for the targeted delivery of chemotherapeutics or for local temperature-induced apoptosis [7]. All these processes could potentially be monitored by MRI, resulting in imaging-guided theranostic systems.

Since SPION-mediated delivery systems can be non-invasively imaged, the precise assessment of drug delivery efficiency in individual patients should be possible. This could be particularly powerful in cancer treatment [8]. In addition, SPIONs are easy to functionalize for combinatory therapy: for example, they can carry therapeutic agents with a high-loading capacity or be labelled with other imaging agents to achieve multimodal imaging [9, 10]. Therefore, SPION-based theranostics have attracted extensive attention image-guided cancer treatment [10, 11].

3.1.2 SPIONs in Electrohydrodynamic Atomization

To avoid the oxidation of magnetite nanoparticles and maintain their colloidal stability against aggregation in suspension or under an external magnetic field, the stabilization of the nanoparticles is crucial. The main strategy used is to graft stabilizers on the surface on the particles. Various stabilisers can be used, including monomeric functional...
groups (carboxylates, phosphates), inorganic materials (silica, gold) or polymers (dextran, Polyethylene glycol, polyvinylpyrrolidone, PVP) [12].

Electro Hydrodynamic atomization (EHDA) can fabricate submicron-to-micron sized fibres or particles encapsulating multiple components using a carrier polymer, for instance aiming for stimuli-responsive drug delivery, or co-delivery of hydrophobic and hydrophilic drugs [13, 14]. Inorganic agents such as SPIONs can also be loaded into electrospayed microparticles or electrospun. Polystyrene fibres loaded with SPIONs were produced by single-fluid EHDA, and could effectively kill cancer cells via magnetic hyperthermia [15]. Rasekh reported a core/shell theranostic prepared by a facile and straightforward co-axial EHDA approach [16]. This particle, composed of a triglyceride tristearin shell and a core loaded with SPIONs, genistein and a fluorophore, could consistently release genistein over 30 h.

3.1.3 Eudragit in pH-Responsive Delivery Systems

pH-responsive polymer systems have been widely used in a number of biomedical applications, particularly in the controlled delivery of bioactive agents such as indomethacin [17], carmofur or lysosome [18]. Due to the acidic environment in the tumour, a variety of pH-responsive delivery systems have been studied for intracellular and extracellular release of bioactive agents [19, 20]. Eudragit S and L have been widely applied as pH-responsive delivery platforms for gastro-resistance and GI targeting [21-23]. It was first marketed in the 1950s by Evonik Industries [24] and have been introduced in USP-NF, British Pharmacopoeia, PhEur and Handbook of Pharmaceutical excipients [21]. Eudragit S and L protects the active ingredient from gastric fluid and will improve drug effectiveness. Eudragit L and S polymers are the preferred choices of coating polymers. They enable targeting specific areas of the intestine. These anionic Eudragit grades dissolve at rising pH values [25]. In addition, different grades can be combined with each other, making it possible to adjust the dissolution pH and thus to achieve required GI targeting for drug [21]. Eudragit offers valuable advantages for enteric coatings include pH-dependent drug release, increase in drug effectiveness, good storage stability, colon targeting and protection of actives sensitive to gastric fluid.

Eudragit L and S polymers are copolymer based on methacrylic acid and ethyl acrylates with representative (Figure 3-1). The various characteristics of Eudragit L and Eudragit
Chapter 3

*pH-Responsive nanocomposite delivery systems*

S include effective and stable enteric coatings with a fast dissolution in the upper bowel, granulation of drug substances in powder form for controlled release, site-specific drug delivery in intestine by combination with Eudragit S grades, variable release profiles [26].

![Chemical structures of Eudragit S100, L100 and L100-55](image)

**Figure 3-1** Structures of Eudragit S100, L100 and L100-55: (A) L100 and S100 are anionic copolymers based on methacrylic acid and methyl methacrylate. The ratio of the free carboxyl groups to the ester groups is approx. 1:1 in L100 and approx. 1:2 in S100. Both typically have molecular weight of approximately 125 kDa; (B) L100-55 is an anionic copolymer based on methacrylic acid and ethyl acrylate. The ratio of the free carboxyl groups to the ester groups is approx. 1:1 and the molecular weight ca. 320 kDa.

### 3.2 Aims and Objectives

In this chapter, SPIONs and carmofur (Figure 3-2), a chemotherapy for colonic cancer, were incorporated into functional polymers via electrohydrodynamic atomization. Our hypothesis is that this kind of formulation can work as a novel orally delivered theranostic, providing imaging guided therapy for colon cancer.

![Structure of carmofur](image)

**Figure 3-2** Structure of carmofur (C_{11}H_{16}FN_{3}O_{3}).
Chapter 3

pH-Responsive nanocomposite delivery systems

To target the colon specifically, Eudragit polymer coating is commonly applied. Owing to the simplicity of and flexibility in the EHDA experimental setup, here we propose to fabricate microparticles and nanofibres encapsulating MRI contrast agents and a model anticancer drug, carmofur. These should prevent SPIONs degradation and undesired drug release in the stomach, allowing theranostics for colon cancer to ultimately be developed.

![Figure 3-3 A schematic illustration of the proposed formulations.](image)

Firstly, SPIONs were synthesized and stabilized. Next, Eudragit was loaded with SPIONs and carmofur via single-fluid EHDA, with the aim of achieving pH-responsive imaging and drug delivery in the small intestine and colon (Figure 3-3). Eudragit L100-55, L100 and S100, which are only soluble in water at pH >5.5, >6.0 or >7.0 respectively, were used to produce formulations. The morphology, physiochemical properties and drug release profiles were investigated, and the acid stability also assessed. To explore imaging guided therapy, we further explored the relationship between proton relaxivity and drug release profiles at different temperatures and pH.
3.3 Experimental

3.3.1 Materials

Chemicals were purchased as follows: sodium hydroxide and hydrous ethanol (Fisher Scientific); sodium chloride, N,N-dimethylacetamide (DMAc), acetone, anhydrous ethanol, polyvinylpyrrolidone (PVP; 40 kDa), hydroxylamine hydrochloride, o-phenanthroline, xanthan gum, citric acid, tetrakethyl orthosilicate (Sigma-Aldrich); Eudragit L100, L100-55 and S100 (Röhm GmbH); and, carmofur (ChemCruz™). Ultrapure water was collected from a Millipore MilliQ system operated at 18.2 MΩ.

3.3.2 Preparation of Solutions for Electrospraying

After a series of preliminary optimization experiments, single fluid electrospraying was achieved using Eudragit S100, L100 and L100-55 to produce microparticles. The recipes, applied voltage and the fluid flow rates are shown in Table 3-1, and experiments performed with a spinneret-to-collector distance of 18 cm. Polymers were dissolved in 10 mL of a solvent system comprising of DMAc and ethanol (2:8 v/v). For loaded-microparticles carmofur and PVP-SPIONs (prepared as described in Section 2.2.1) were then added to the Eudragit L100 or S100 solutions respectively, with sonication for 20 min.
Table 3-1 Formulations of Eudragit loaded with carmofur, or with carmofur/ PVP-SPIONs and parameters for electrospraying

<table>
<thead>
<tr>
<th>No</th>
<th>Polymer (w/v)</th>
<th>Carmofur (w/w)</th>
<th>PVP-SPIONs (w/w)</th>
<th>Flow rate (mL/h)</th>
<th>Voltage (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100-C</td>
<td>3.5% Eudragit S100</td>
<td>10%</td>
<td>-</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>L100-C</td>
<td>3% Eudragit L100</td>
<td>10%</td>
<td>-</td>
<td>0.6</td>
<td>22</td>
</tr>
<tr>
<td>L100-55-C</td>
<td>3% Eudragit L100-55</td>
<td>10%</td>
<td>-</td>
<td>0.6</td>
<td>22</td>
</tr>
<tr>
<td>S100-C-SPIONs</td>
<td>3.5% Eudragit S100</td>
<td>10%</td>
<td>20%</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>L100-C-SPIONs</td>
<td>3%</td>
<td>10%</td>
<td>20%</td>
<td>0.6</td>
<td>22</td>
</tr>
<tr>
<td>L100-55-C-SPIONs</td>
<td>3%</td>
<td>10%</td>
<td>20%</td>
<td>0.6</td>
<td>22</td>
</tr>
</tbody>
</table>

(a; a: % w/w ratio of carmofur or SPIONs with respect to polymer)

Preparation of solutions for electrospinning

A 12% (w/v) solution of Eudragit L100 or 10% (w/v) solution of Eudragit S100 was prepared in a mixture of DMAc and ethanol (2:8 v/v) and stirred vigorously for 24 h. For loaded-fibres carmofur (to give final concentrations of 12 or 10 mg/mL) and PVP-SPIONs (at final concentrations of 24 or 20 mg/mL) were then added to the Eudragit L100 or S100 solutions respectively, with sonication for 20 min.

Preparation of microparticles or nanofibres

The EHDA processes were given in Section 2.3.1. The experimental parameters for electrospinning are illustrated in Table 3-1. For electrospinning, the flow rate was 1.0 mL/h, and the applied voltage and the distance from the spinneret to collector were set to 16 kV and 15 cm, respectively.

3.3.3 Formulation Characterization

Morphology

The morphology of both Eudragit microparticles and fibres are characterised using SEM, the protocols are given in Section 2.4.1.
Physicochemical Characterisation

Eudragit microparticles and fibres were characterised using XRD, DSC, TGA and FTIR as described in Section 2.4.2.

Dynamic light scattering (DLS) measurement were given in Section 2.4.3.

3.3.4 Stability Assay

Stability studies were carried by dispersing 10 mg of the formulation composites or 2 mg of PVP-SPIONs in 25 mL of an aqueous HCl solution (pH 1.5). Experiments were carried out in a shaking incubator (100 rpm) at 37 °C for 2 h. The resulting solutions were centrifuged for 15 min (13,200 rpm) to sediment any undissolved fibres or particles. 5 mL samples of the supernatants were removed and neutralized with a few drops of aqueous 0.2 M NaOH. 0.9 mL of the neutralized sample was added to 0.3 mL of a 10 % (w/v) hydroxylamine hydrochloride solution in water and 0.6 mL of an aqueous 0.2 % (w/v) o-phenanthroline solution. Finally, UV-vis spectra were recorded on an Agilent Cary 100 spectrophotometer over the wavelength range 250 to 800 nm.

3.3.5 In vitro Drug Release

Firstly, loading capacity and encapsulation efficiency is measured based on protocol in Section 2.5.1.

A 50 mL suspension of the formulations (~ 0.5 mg/mL) was incubated in a pH 1.5 HCl solution for 1 h, and then transferred to pH 6.5 or 7.4 phosphate-buffered saline (PBS). Experiments were undertaken in a shaking incubator (100 rpm) at 37 °C. 1 mL aliquots were withdrawn from the dissolution medium at predetermined time points and filtered through a PVDF-type syringe filter (0.22 μm). To maintain a constant volume, 1 mL of fresh pre-heated buffer was added to the dissolution vessel. The filtrates were centrifuged for 15 min (13,200 rpm) to remove any SPIONs, and then analyzed with an Agilent Cary 100 spectrophotometer. Carmofur quantifications were performed at $\lambda_{\text{max}}$ of 262 nm. Dilutions were undertaken when necessary to bring concentrations into the linear range of the calibration curve. Experiments were performed in triplicate and the results are reported as mean ± standard deviation (S.D.).
3.3.6 Relaxivity Behaviour Studies

Proton relaxivity ($r_{1,2}$) were measured as described in Section 2.4.3.1

Proton Relaxivity Monitoring

To monitor changes in proton relaxivity with pH and temperature, a dispersion of approximately 10 mg of each formulation composite in 8 mL of a 0.1 % (w/v) aqueous xanthan gum solution was placed into a 10 mm-diameter nuclear magnetic resonance (NMR) tube, which was held at 37 °C. The transverse relaxation time ($T_2$) was directly monitored over 3 h. At predetermined time points, 0.3 mL of suspension was taken from the NMR tube, diluted, and filtered through a PVDF-type syringe filter (0.22 μm). To measure the free iron concentration, suspensions were analysed by inductively coupled plasma mass spectrometry (ICP-MS) after hot nitric acid digestion. To measure the carmofur concentration, centrifugation (13,200 rpm for 10 min) was conducted to remove the SPIONs, and the supernatant analysed on an Agilent Cary 100 spectrophotometer. All experiments were performed in triplicate and the results are reported as mean ± S.D.

Drug Release Predicting

In a set of experiments to predict drug release, dispersions of fibre samples at different concentration (0.5, 1.0 and 2 mg/mL) in 0.1% w/v xanthan gum buffer (n=3) were placed into 10 mm NMR tubes. The transverse relaxation time was monitored at 37 °C for 3 h. At selected time points, 0.3 mL aliquots were taken from the NMR tube and the [Fe] and carmofur content in each aliquot quantified as above.

3.4 Results

3.4.1 Synthesis and Stabilization of SPIONs

SPIONs were synthesized as the MRI contrast agent for our theranostic formulations as described in Section 2.1.1. The coprecipitation technique was employed to synthesize these. This method is a simple and efficient chemical route to obtain iron oxide particles by aging a stoichiometric mixture of ferrous and ferric salts in an aqueous basic medium (Equation 3-1) [27].
Magnetite (Fe₃O₄) is relatively stable in its bulk and solid states, but in aqueous solution, it can be transformed into maghemite (γ-Fe₂O₃) (Equation 3-2) [27].

\[
\text{Fe}_3\text{O}_4 + 2\text{H}^+ \rightarrow \gamma-\text{Fe}_2\text{O}_3\text{Fe}^{3+} + \text{H}_2\text{O}
\]  
Equation 3-2

### 3.4.1.1 SPIONs Characterization

**XRD**

The XRD data (Figure 3-4) confirm the synthesis of magnetite. The reflections at 30.2, 35.5, 43.7, 53.6, 57.1 and 62.9° can be readily indexed to the (022), (311), (004), (333), (115), and (044) planes of reverse spinel Fe₃O₄ (ICSD entry 77592) [28].

![Figure 3-4 XRD patterns of SPIONs (red), PVP stabilized magnetite nanoparticles (PVP-SPIONs, green), and PVP (purple).](image)

**UV**

UV/vis spectra further suggested the synthesis of SPIONs (Figure 3-5) with a maximum absorption around 220 nm, in accordance with the literature [29].
Figure 3-5 UV-vis spectrum of SPIONs and PVP-SPIONs in water.

**IR Spectra**

FTIR spectra also provided evidence for the presence of Fe-O bonds, with stretches at 547 cm\(^{-1}\) (Figure 3-6).

Figure 3-6 IR spectrum of PVP, SPIONs and PVP-SPIONs.
3.4.1.2 SPIONs Stabilization

Polyvinylpyrrolidone (PVP) is a hydrophilic, biocompatible, polymer. PVP coating onto SPIONs surfaces prevents their agglomeration, giving rise to monodisperse particles. Here PVP was physiosorbed onto the surface of magnetite nanoparticles after stirring via electrostatic and hydrogen bonding interactions, to generate steric repulsion and provide colloidal stability. The strong UV absorption of the SPIONs between 200 to 320 nm was not present for the PVP-SPIONs, suggesting successful coating by PVP (Figure 3-5).

FTIR

The FTIR spectra confirm that PVP binds to the surface of magnetite (Figure 3-6). The spectra of both PVP and the PVP-SPIONs show the presence of C-H absorptions from PVP at around 1450 cm\(^{-1}\) (CH\(_2\) and CH\(_3\) bending) and 2900 cm\(^{-1}\)(C-H stretching). For PVP, the band at 1283 cm\(^{-1}\) can be assigned to the C=N stretching vibration; this vibration showed a red shift after being physiosorbed on the surface of the SPIONs (1180 cm\(^{-1}\)) indicating the C-N bond are more stable after coordination with the iron atoms. Conversely, the C=O stretching vibration displays a blue shift after PVP binds to the magnetite nanoparticles. For PVP the C=O vibration presents as a strong peak at 1650 cm\(^{-1}\), while in the PVP-SPIONs it sits at around 1730 cm\(^{-1}\). This blue shift suggests interactions between the carbonyl group of PVP and iron ions in the magnetite nanoparticles. Another strong peak around 1610 cm\(^{-1}\) in the spectrum of the PVP-SPIONs also suggests coordination between the lone-pair electrons in the carbonyl groups of PVP with iron ions on the surface of the nanoparticles.

Morphology

Figure 3-7 depicts TEM images of the PVP stabilized magnetite nanoparticles (PVP-SPIONs), which have diameters below 20 nm. XRD patterns of PVP and PVP-SPIONs are shown in Figure 3-4. The SPION Bragg reflections from reverse spinel Fe\(_3\)O\(_4\) are still present. In addition, there is a broad peak at 18-22° 2\(\theta\), which corresponds to that of PVP. All of these observations are consistent with successful coating of the SPIONs.
Thermogravimetric Analysis (TGA)

TGA was also carried out for the PVP-SPIONs and SPIONs (Figure 3-8). TGA data of the non-stabilised SPIONs revealed a mass loss of 2.4 wt % between 40 and 170 °C, reflecting the gasification of solvent (e.g. water) and surface hydroxyl groups. The material subsequently loses a mass of 1.8 wt % between 170 and 400 °C, which might be caused by the transformation of the Fe₃O₄ phase into γ-Fe₂O₃, and then remained constant between 400 and 500 °C, as expected [30]. In contrast, TGA of PVP alone reveals a weight loss of 90 wt % between 170 and 500 °C as a result of the decomposition of its hydrocarbon chains. For the PVP-SPIONs, the TGA curve displays a mass loss of 3.4 wt % between 40 and 170 °C, which is due to the evaporation of the solvent and physisorbed water, and a loss of 6.2 wt % between 170 and 500 °C. The latter can be attributed to the degradation of the PVP stabiliser; therefore, the SPIONs comprise around 90 wt % of the PVP-SPIONs mass.
Colloidal stability

To further explore the colloidal stability of the SPIONs, the hydrodynamic diameters of the PVP-SPIONs were measured over one hour in ethanol, using dynamic light scattering (DLS). The DLS results (Figure 3-9) showed PVP-SPIONs are relatively stable in water, unlike the non-stabilized SPIONs, which do not yield a stable colloidal suspension for DLS measurement. After one hour, the hydrodynamic diameter of the PVP-SPIONs increases in size only slightly (Table 3-2).
Table 3-2 The hydrodynamic size and PDI of PVP-SPIONs in ethanol (~1 mg/mL)

<table>
<thead>
<tr>
<th>Particle</th>
<th>Time</th>
<th>Hydrodynamic diameter / nm (n=3)</th>
<th>PDI (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP-SPIONs</td>
<td>0 h</td>
<td>220 ± 2</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>331 ± 8</td>
<td>0.29 ± 0.04</td>
</tr>
</tbody>
</table>

Compared to the actual size measured via TEM images, the hydrodynamic diameter of PVP-SPIONs determined via DLS is much larger. This is due to the fact particles are hydrated in suspension, and both particles and bound water are measured. Furthermore, hydrodynamic radius obtained via DLS takes into account both translational and rotational motion of nanoparticles in suspended medium. As the hydrodynamic radius of gyration of PVP-SPIONs was much higher compared to the distance traversed during translational motion, a higher particle size value measured using DLS technique is expected [31]. Furthermore, the direct intensity size distribution may also inherently be weighted to larger size particles, due the fact that the scattering intensity is proportional to size [32]. Hence, for our particles, the sizes obtained from intensity-based measurement were overestimated, but the DLS results can still provide an insight to the behaviour of our particles in aqueous phase.

3.4.2 EHDA Formulation

3.4.2.1 Blank Eudragit Microparticles

The model drug used in the theranostic here is carmofur, which has been employed as an adjuvant chemotherapy for colorectal cancer patients [33]. Before encapsulating MRI contrast agents, we first confirmed the fabrication of composites loaded with carmofur.

Morphology

Figure 3-10 shows the morphologies of the microparticles produced.
Figure 3-10 SEM images and size distributions of electrosprayed microstructures A) S100-C, B) L100-C, C) L100-55-C; sample nomenclature as described in Table 3.1. Diameters calculated by measuring at least 100 particles.

The Eudragit S100 microparticles emerged as irregularly shaped pseudo-spherical particles with rough surfaces and a broad size distribution, with a mean diameter of 303 ± 293 nm. In contrast, the Eudragit L100 particles are more homogenous (mean diameter 301 ± 217 nm), with some tiny fibres on the surface. The L100-55 microparticles have a smooth surface, but some small fibres emerged as well, possibly due to the higher MW of Eudragit L100-55 (mean diameter 573 ± 358 nm).

3.4.2.2 Particles Containing Carmofur and SPIONs

Morphology

SEM images of electrosprayed particles containing both carmofur and SPIONs are depicted in Figures 3-11. Compared to the particles loaded with carmofur only (Figure
3-10), those containing carmofur and PVP-SPIONs display a narrower size distribution, as given in Figure 3-12.

![Figure 3-11 SEM images of electrosprayed microstructures: A) S100-C-SPIONs, B) L100-C-SPIONs, C) L100-55-C-SPIONs. Sample labels as detailed in Table 3-2.](image)

The electrosprayed Eudragit S100 and L100 materials loaded with carmofur and PVP-SPIONs (S100-C-SPIONs and L100-C-SPIONs) emerge as irregularly-shaped spherical particles with rough surfaces, and average diameters of 863 ± 333 nm and 419 ± 176 nm. The Eudragit L100-55 particles containing PVP-SPIONs and carmofur (L100-55-C-SPIONs) are different, taking the form of small 1-dimensional rod shaped particles with a mean width of 290 ± 117 nm and length of 902 ± 411 nm. The size of particles loaded with both PVP-SPIONs and carmofur is larger compared to SPIONs free particles.
Physiochemical Characterization

XRD

The XRD patterns of the raw materials and different formulations are presented in Figure 3-13. The patterns of all the electrospayed formulations loaded with carmofur display only a broad peak between 3-40 °, indicating a lack of crystalline structure. This is in stark contrast to the pattern of raw carmofur, which has numerous sharp Bragg reflections owing to the crystalline nature of the pure drug. These patterns reflect the amorphous nature of the Eudragit S100, L100, L100-55 and carmofur loaded formulations. The reflections of PVP-SPIONs at 30.2, 35.5, 43.7, 53.6, 57.1 and 62.9 ° can be clearly observed in the particles, indicating the successful encapsulation of PVP-SPIONs in the formulations.
Figure 3-13 XRD patterns of the raw materials and electrosprayed formulations.

TGA

The TGA traces of the raw materials (Figure 3-14) show that carmofur degrades from approximately 110 to 300 °C, while the major weight loss of the raw L100 and S100 polymers began at 300 and ended at around 460 °C, where less than 5% of the original mass remains. The TGA curves of the L100-C-SPIONs, L100-55-SPIONs and S100-C-SPIONs microparticles all display similar multistage decomposition processes. The trace mass loss of about 3 % before 110 °C in can be attributed to the loss of solvent (e.g. physiosorbed water).
Figure 3-14 TGA profiles of (A) raw materials, (B) S100-C-SPIONs (n=3), (C) L100-C-SPIONs (n=3), (D) L100-55-C-SPIONs (n=3).

The loss of molecular fragments of carmofur can be associated with two decomposition stages over the temperature ranges of 110 to 160 and 220 to 300 °C. At the same time, a small proportion of Eudragit is degraded between 110 to 300 °C. The last stage of decomposition between 330 to 460 °C causes a weight loss of around 54%, due to depolymerization of Eudragit [34, 35].

From the TGA analysis of the microparticles fabricated from three independent electrospray processes (n=3), we can see good reproducibility, with the iron oxide content at around 20% w/w (21.9 ± 2.4 % w/w (S100-C-SPIONs), 19.4 ± 1.4 % (L100-55-C-SPIONs), 21.6 ± 0.6 % (L100-C-SPIONs). This is somewhat higher than the theoretical loading (15.4 % w/w). This discrepancy arises because there is a small proportion of residual polymer (around 3%) which remains at 500 °C.
Chapter 3

pH-Responsive nanocomposite delivery systems

DSC

The DSC profiles of carmofur and Eudragit are depicted in Figure 3-15A. Only carmofur exists as a crystalline material, with two sharp endothermic melting events visible at ca. 114 and 115 °C. This is consistent with the literature [36, 37], and agrees with the XRD data. The DSC curves of the Eudragit polymers display broad endothermic peaks centred at around 80 °C, as a result of solvent evaporation [35]. No obvious glass transition temperature can be seen.

The absence of the carmofur melting endotherm in the DSC curves of the drug- and SPIONs-loaded formulations demonstrates that the active ingredient exists as amorphous solid dispersions in these formulations, as a result of the very rapid drying which occurs during electrospraying (Figure 3-15B) [35, 37]. Broad endotherms below 110 °C can be seen, owing to solvent loss. Again, these findings are fully consistent with the XRD data.

Figure 3-15 DSC data for (A) raw polymer and PVP-SPIONs, and (B) the electrosprayed microparticles and carmofur.

3.4.2.3 Nanofibres Prepared by Electrospinning

When explored the $T_2$ relaxometry of SPIONs-loaded microparticles, we found that the maximum transverse relaxivity ($r_2$) that can be obtained from Eudragit L100 and S100 microparticles (~ 60 mM$^{-1}$·s$^{-1}$) are much higher than that from Eudragit L100-55 (~ 30 mM$^{-1}$·s$^{-1}$). This can be related to the higher MW of Eudragit L100-55, preventing the interaction between water protons and SPIONs, which will be detailed explained in Section 3.4.5.2. Hence, here we only use Eudragit L100 and S100 to prepare fibres.
Morphology

The electrospun L100 and S100 fibres loaded with SPIONs and carmofur (L100-C-SPIONs-F and S100-C-SPIONs-F) have uniform linear morphologies and smooth surfaces, with mean diameters of 645 ± 225 and 454 ± 133 nm respectively (Figure 3-16 and 3-17).

Figure 3-16 SEM images of (A, B) L100-C-SPIONs-F and (C, D) S100-C-SPIONs-F fibres.

The larger diameter of the former might be due to the higher polymer concentration using for electrospinning. The concentration of Eudragit S100 (10%) is lower than that of Eudragit L100 (12%) in the working solution, because a higher concentration of Eudragit S100 causes a gel-like substance to form on the spinneret, and subsequent clogging of the needle.
**Figure 3-17** Size distribution of (A) L100-C-SPIONs-F and (B) S100-C-SPIONs-F fibres loaded with PVP-SPIONs and carmofur.

**Physicochemical Characterization**

**XRD**

XRD patterns (Figure 3-18) confirm the encapsulation of SPIONs with a cubic structure in the fibres. However, the characteristic reflections of carmofur (Figure 3-13) cannot be seen in the fibres’ patterns. This demonstrates that the carmofur is present in the amorphous form in the fibres, which is consistent with the results for electrosprayed microparticles above, and also the literature [35, 37].

**Figure 3-18** XRD patterns of the SPIONs, PVP-SPIONs and L100-C-SPIONs-F and S100-C-SPIONs-F fibres.
TGA

The TGA curves of L100-C-SPIONs-F and S100-C-SPIONs-F fibres (Figure 3-19) have same multistage decomposition patterns with electrosprayed microparticles (Figure 3-14). From the TGA of the fibres, we can calculate that the iron oxide content is around 20 % w/w (17.3 ± 0.34 % w/w for L100-C-SPIONs-F and 20.6 ± 0.26 % for S100-C-SPIONs-F fibres; mean ± S.D., n = 3). Similar to the electrospayed microparticles, the iron oxide content calculated is higher than the theoretical loading (15.4 % w/w for L100-C-SPIONs-F and 17.9 % w/w for S100-C-SPIONs-F fibres). This discrepancy arises because there is a small proportion of residual polymer (around 3%) which remains at 500 °C, and is consistent with the TGA profile of the microparticles (Figure 3-14).

![TGA curves of S100-C-SPIONs-F and L100-C-SPIONs-F (n = 3).](image)

Figure 3-19 TGA curves of S100-C-SPIONs-F and L100-C-SPIONs-F (n = 3).

DSC

DSC data for the fibres are displayed in Figure 3-20.
Pure carmofur clearly exists as a crystalline material, with two sharp endothermic melting peaks visible at ca. 114 and 115 °C, consistent with the literature [36, 37]. The L100-C-SPIONs-F and L100-C-SPIONs-F formulations show broad shallow endotherms between 40 to 80 °C, which can be ascribed to a loss of solvent (ethanol, DMAc, or adsorbed water). The absence of the carmofur melting endotherm in the DSC curves of the drug-loaded formulations demonstrates that it is present in the form of an amorphous solid dispersion, which is same with the electrosprayed microparticles. This is also consistent with the literature [36, 37], and agrees with the XRD data.

### 3.4.3 Acid Stability Assessment

The parent SPIONs are sensitive to acidic environments such as that found in the stomach, resulting in their dissolution and loss of magnetic properties. Their encapsulation in Eudragit S100, L100, or L100-55 should solve this problem. These polymers are not soluble under the pH conditions of the stomach, and thus could protect the magnetic particles from digestion. In order to test the stability of the formulations in the gastrointestinal (GI) tract pH environment, the formulations were incubated in pH 1.5 hydrochloric acid at 37 °C (100 rpm) for two hours.
3.4.3.1 Acid Stability of Electrosprayed Microparticles

Morphology

The SEM images of the microparticles after immersion in pH 1.5 HCl further confirmed the stability of the formulations (Figure 3-21).

![SEM images of microparticles after pH 1.5 HCl immersion](image)

Figure 3-21 SEM images after immersion in pH 1.5 HCl for 2h. Images are shown for (A) S100-C-SPIONs, (B) L100-C-SPIONs and (C) L100-C-SPIONs, labels as described in Table 3-2.

For the S-C-SPIONs and L100-C-SPIONs, the shapes and size remain similar to those of the original particles, with a mean diameter of 823 ± 349 nm and 676 ± 293 nm, respectively (Figure 3-22A, B). For L100-55-C-SPIONs, the microspheres change from rod-shaped to spherical and grow in size with mean diameter of 812 ± 339 nm (Figure 3-22C). This might be due to the swelling of the L100-55-C-SPIONs particles in HCl solution.

![Size distribution of microparticles after pH 1.5 HCl immersion](image)

Figure 3-22 Size distribution of the microparticles after immersion at pH 1.5 for 2h. Diagrams are shown for (A) S100-C-SPIONs, (B) L100-C-SPIONs and (C) L100-C-SPIONs.
[Fe] Release

Any release of iron was monitored using an o-phenanthroline colorimetric assay. The $\lambda_{\text{max}}$ of ferrous tris-o-phenanthroline in hydrochloric acid is 512 nm. The UV/vis absorption spectra of the PVP-SPIONs and carmofur loaded electrosprayed particles are presented in Figure 3-23.

![Figure 3-23 UV/vis absorption spectra after immersion in a pH 1.5 HCl solution for 2 h.](image)

The spectra reveal that [Fe] after incubation of the SPION loaded microparticles was less than 2 mg/L, indicating less than 1 wt.% of the total SPIONs content was degraded in the loaded microparticles. This concentration is significantly lower than the [Fe] released from bare PVP-SPIONs at equivalent concentrations (between 2-10 mg/L, equating to up to 17 wt.% degradation).

3.4.3.2 Acid Stability of Electrospun Fibres

Morphology

The acid stability of the electrospun fibres was also investigated. SEM images (Figure 3-24 A-D) demonstrate the stability of the L100/Carmofur/SPIONs and S100/Carmofur/SPIONs fibres: the morphology of the fibres appears largely unaffected after exposure to the acidic conditions, and the fibre size also remains similar, at 612 ±
227 nm for the L100-C-SPIONs-F and 521 ± 166 nm for the L100-C-SPIONs-F fibres (Figure 3-25).

Figure 3-24 The stability of PVP-SPIONs and the fibres after immersion at pH 1.5 for 2 h. SEM images are shown for (A, B) S100-C-SPIONs-F N and (C, D) L100-C-SPIONs-F fibres, labelled as described in Table 3-2.

Figure 3-25 Size distribution of (A) the L100-C-SPIONs-F and (B) S100-C-SPIONs-F fibres after suspension in pH 1.5 HCl for 2 h.
[Fe] Release

UV/vis absorption spectra of the PVP-SPIONs and fibres after 2 h are shown in Figure 3-25. The concentration of iron in solution was below 1 mg/L, less than 1 wt% of the total SPION content in the fibres and much lower than with the same amount of bare PVP-SPIONs (blue, Figure 3-26). These results show that the Eudragit matrix can protect the SPIONs from degradation in an acidic environment. Overall, both electrospayed microparticles and electrospun fibres can effectively protect SPIONs from degradation.

![Graph showing absorption spectra](image)

Figure 3-26 The results of colorimetric assays to quantify the amount of Fe present in solution after 2 h.

### 3.4.4 In vitro Drug Release

#### 3.4.4.1 LC and EE

To calculate the encapsulation efficiency (EE) and loading capacity (LC) of carmofur, the formulations were dissolved in ethanol and after the SPIONs were removed by filtration the concentration of carmofur was measured by UV spectroscopy at 255 nm (see in Section 2.5.1). The LC of the S100, L100 and L100-55-C-SPIONs formulations are $8.1 \pm 0.4$, $8.3 \pm 0.2$, and $8.7 \pm 0.9\%$ (mean $\pm$ S.D., $n = 5$). The corresponding EEs
are 96.8 ± 6.4, 97.3 ± 3.3, 102.0 ± 8.9% (mean ± S.D., n = 5). The EE is largely consistent among these three formulations, and similar to previous work in our lab [38]. Usually EHDA can achieve very high encapsulation efficiency [38, 39] and the EE value of L100-55-C-SPIONs is above 100%, which might due to a small proportion of PVP-SPIONs were sedimented on the bottom of syringe during the electrospray process, thus the drug ratio in fibres were slightly increased.

The carmofur loadings of the L100 and S100 fibres are calculated to be 7.5 ± 0.4 % and 8.0 ± 0.4 % (mean ± S.D., n = 3), with encapsulation efficiencies of 96.1 ± 4.9 % and 100.1 ± 5.4 % (mean ± S.D., n = 3) respectively. Similar to electrosprayed microparticles, electrospun fibres also can achieve very high encapsulation efficiency as reported in literature [38, 39].

### 3.4.4.2 In vitro Release

To mimic oral delivery, the in vitro release profiles of the microparticles or fibres were explored in pH 1.5 HCl solution for 1 h and subsequently in pH 6.5 or 7.4 PBS. These experiments were employed because the pH varies widely though the GI tract: the gastric pH is highly acidic (range 1.0-2.5), while the mean pH in the proximal small intestine, colon and terminal ileum is 6.6, 7.0 and 7.5 [40]. Thus, buffers with different pH were employed to simulate the different environments along the GI tract. As the UV-vis absorption of magnetite nanoparticles can interfere with the λ_{max} of carmofur (262 nm), the aliquots taken were centrifuged to remove all the magnetite nanoparticles before measurements were taken.

**Microparticles**

The drug release profiles of the microparticles loaded with PVP-SPIONs and carmofur are depicted in Figure 3-27. Around 40% of the total carmofur loading (37.1 - 43.2 %) was released from the three microparticles in HCl solution after first hour. There is then a subsequent burst of release when the formulations are transferred to close-to neutral pH. At pH 7.4, S100-C-SPIONs display a faster release than at pH 6.5, while the drug release profile at these two pHs was similar for the other two formulations. This is because Eudragit S100, L100, L100-55 become soluble at different pHs: 7, 6, and 5.5, respectively. At pH 6.5, the S100-C-SPIONs remained insoluble, hence the drug can only reach the dissolution medium through diffusion or swelling of the polymer and the
permeation of water into the centre of the fibres, which slowed the carmofur release rate. After 3 hours, more than 90% of carmofur was released from all the formulations, again expect the S100-C-SPIONs, which achieve 90% release in 6 hours.

Figure 3-27 Drug release profiles of the electrosprayed particles after 1h at pH 1.5 and then immersion at pH 7.4 (black) or 6.5 (red) PBS. (A) S100-C-SPIONs, (B) L100-C-SPIONs, (C) L100-55-C-SPIONs. Data are given from three independent experiments as mean ± S.D.
**Fibres**

The carmofur release profiles of L100-C-SPIONs-F and S100-C-SPIONs-F fibres are similar to the microparticle formulations, as shown in Figure 3-28.

![Figure 3-28](image)

**Figure 3-28** Plots showing the release of carmofur from the L100/SPIONs/Carmofur and S100/SPIONs/Carmofur fibres. Data are given from three independent experiments as mean ± S.D.

Both sets of fibres demonstrated release of around 35 % (31.9 – 36.6 %) of the drug content in the acidic environment, followed by rapid release at close to neutral pH. The L100-C-SPIONs-F exhibit a similar release pattern at both pH 6.5 and pH 7.4, with more than 90 % release within 2 h and 100 % release after 24 h. For the S100-C-SPIONs-F, carmofur release reaches 80 % after 2 h at pH 7.4, while it takes longer (approximately 4 h) to reach this point at pH 6.5.

The carmofur released from the electrosprun fibres in both HCl solution and PBS is slightly lower than the amount freed from the analogous electrosprayed microparticles. The difference in release profile between microparticles and nanofibre might be related to the distinct micro/macro structures of fibre and microparticles. Microparticle possesses a very high surface-to-volume ratio and thus facilitate the interaction between water and inner polymer matrix, as well as drug diffusion into solutions. While fibre is presented as membrane-like compact blends, water access into inner layer might take longer.
3.4.4.3 Size Distribution Profiles

DLS size distribution profiles further support the pH-sensitive dissolution properties of the composites. After the 24 h drug release study, the particle size of the solid material in the dissolution medium was measured, and the results are given in Figure 3-29 and Table 3-3.

Figure 3-29 Particle size distribution curves of (A) S100-C-SPIONs, (B) L100-C-SPIONs (C) L100-55-C-SPIONs, (D) L100/-C-SPIONs-F and (E) S100/-C-SPIONs-F at various pHs (~ 0.5 mg/mL). The data were represented as the average of three independent DLS measurements.

The L100 particles and fibres dissolve at both pH 6.5 and 7. According to [41], the amphiphilic Eudragit copolymer can form micellar structures composed of a hydrophobic core and a shell with ionized carboxylate units. Hence, micelles appear to form with a mean size of 575 ± 27 nm and 358 ± 27 nm (n = 3) for the microparticles, or 591 ± 32 nm and 380 ± 11 nm for the fibres. But there is also a possibility that other aggregates are formed. In contrast, at pH 5.5 the mean particle size of the L100 fibre and microparticle suspensions were 3049 ± 41 and 3080 ± 140 nm, respectively, suggesting the polymer remains insoluble.
Table 3-3 The hydrodynamic size and PDI of the formulations at different pHs. The measurement was taken using of the release medium after 24 h. The concentration was ca. 0.5 mg/mL.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>pH</th>
<th>Hydrodynamic diameter / nm (n=3)</th>
<th>PDI (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.4</td>
<td>380 ± 11</td>
<td>0.53 ± 0.02</td>
</tr>
<tr>
<td>L100-C-SPIONs-F</td>
<td>6.5</td>
<td>591 ± 32</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>3049 ± 41</td>
<td>0.20 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>458 ± 31</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>S100-C-SPIONs-F</td>
<td>6.5</td>
<td>2959 ± 176</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>358 ± 27</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>L100-C-SPIONs</td>
<td>6.5</td>
<td>575 ± 27</td>
<td>0.84 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>3080 ± 53</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>406 ± 13</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>S100-C-SPIONs</td>
<td>6.5</td>
<td>2782 ± 130</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>837 ± 103</td>
<td>0.73 ± 0.10</td>
</tr>
<tr>
<td>L100-55-C-SPIONs</td>
<td>6.5</td>
<td>1022 ± 100</td>
<td>0.84 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>3179 ± 182</td>
<td>0.26 ± 0.15</td>
</tr>
</tbody>
</table>

Eudragit S100 microparticles and fibres displayed analogous results in DLS with a mean size of 458 ± 31 nm at pH 7.4 and 2959 ± 176 nm at pH 6.5 for S100-C-SPIONs-F fibres, and 406 ± 13 nm at pH 7.4 and 2782 ± 130 nm at pH 6.5 for S100-C-SPIONs particles (n = 3). Even though the L100-55-C-SPIONs microparticles are soluble at pH 5.5, 6.5 and 7.4, the hydrodynamic diameter shows an analogous trend, with a lower hydrodynamic diameter at high pH (837 ± 103 nm at pH 7.4, 1022 ± 100 nm at pH 6.5) and higher hydrodynamic diameter at low pH (3179 ± 182 nm at pH 5.5). The size of L100-55-C-SPIONs at all three pHs are much higher than that of Eudragit L100 and S100, which can be related to the longer chain of Eudragit L100 as a result of higher molecular weight compared to Eudragit S100 and L100. Thus the micellar structures formed after dissolution possess larger size in buffers.

### 3.4.4.4 Release Kinetics

**Korsmeyer Peppas Model**

To understand better the mechanism of drug release, the Korsmeyer Peppas model (Equation 2-2) was fitted to the fibre data in the pH 7.4 or 6.5 buffers. For the
microparticles, the release is more rapid (around 50% after 1 hour) meaning it is not possible to fit the Peppas model (see in Section 2.8.1.1) [42]. As shown in Table 3-4 and Figure 3-30, all the fibre release data can be fitted well with the model. All the exponents at both pHs are in the range 0.45 to 0.89, indicating drug release occurs through a combination of matrix swelling and drug diffusion.

Figure 3-30 Fitting of the Peppas model to drug release from (A) L100-C-SPIONs-F, pH 7.4; (B) L100-C-SPIONs-F, pH 6.5; (C) S100-C-SPIONs-F, pH 7.4; (D) S100-C-SPIONs-F, pH 6.5.

Table 3-4 The results of fitting the Peppas model to carmofur release from the electrospun fibres.

<table>
<thead>
<tr>
<th>Fibres</th>
<th>pH</th>
<th>Peppas model</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>L100-C-SPIONs-F</td>
<td>7.4</td>
<td>$Q_t = 73.8 t^{0.75}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>$Q_t = 52.7 t^{0.53}$</td>
<td>0.98</td>
</tr>
<tr>
<td>S100-C-SPIONs-F</td>
<td>7.4</td>
<td>$Q_t = 61.6 t^{0.75}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>$Q_t = 35.6 t^{0.54}$</td>
<td>0.99</td>
</tr>
</tbody>
</table>
3.4.5 Relaxivity and Carmofur Release Monitoring

3.4.5.1 Proton Relaxation Behaviours for PVP-SPIONs

SPIONs are effective MRI contrast agents, providing negative contrast by decreasing the transverse relaxation time of local protons. Firstly, the relaxivity of PVP-SPIONs was calculated according to **Equation 2-1**. The longitudinal ($r_1$) and transverse relaxivity ($r_2$) of the PVP-SPIONs were 3.58 and 63.1 mM$^{-1}$·s$^{-1}$ at 37 °C (Figure 3-31). The $r_2/r_1$ ratio is more than 10, confirming negative MRI contrast behaviour, agreeing with the literature[43, 44].

![Figure 3-31](image)

**Figure 3-31** The longitudinal and transverse relaxivity profiles of the PVP-SPIONs.

3.4.5.2 $r_2$ and Corresponding Carmofur Release

To explore the SPIONs’ contrast behaviour after encapsulation within the formulations, the relaxivity ($r_2$) was measured in buffers at different pH with 0.1 % w/v xanthan gum and calculated according to **Equation 2-1**. The fibres and particles were continuously incubated in the buffer to monitor the change of contrast behaviour with time. The carmofur release profile was also monitored to elucidate any relationship between the drug release and proton relaxativity.
Electrosprayed Microparticles

Figure 3-32 The proton transverse relaxation and corresponding carmofur release profiles of microparticles in 0.1% xanthan gum. A) proton relaxivity, B) carmofur release of S100-C-SPIONs; C) proton relaxivity, D) carmofur release of L100-C-SPIONs; E) proton relaxivity, F) carmofur release of L100-55-C-SPIONs.

For S100-C-SPIONs, it can be seen that the transverse relaxivity gradually increases at pH 7.4 but remained very low at pHs below 7 (Figure 3-32A). As the formulations are can soluble at pH > 7, the PVP-SPIONs were still inside the particles at the lower pH
values after 3 hours of immersion as shown in photographs (Figure 3-33), and therefore the relaxivity remains very low due to their being largely unable to interact with water protons [45].

The drug release from the S100-C-SPIONs (Figure 3-32B) displays very rapid release at pH 7.4, reaching to around 90 % in the first hour. At the lower pHs, where the polymer remains insoluble, the materials essentially act as diffusion-controlled matrix systems. Owing to the amorphization of drug and the large surface to volume ratio of the microparticles, the percentage of carmofur released reached 60 % even in a pH 4 buffer.

Figure 3-33 Photographs of the microparticles in PBS buffer at different pH after 3 hour immersion, as labelled. (A) L100-C-SPIONs dissolve in PBS at both pH 6.5 or 7.4 but not pH 4.0 or 5.5, (B) L100-55-C-SPIONs dissolve in PBS at both pH 6.5 or 7.4 but not pH 4.0 or 5.5 while (C) S100-C-SPIONs dissolves at pH 7.4 but not pH 6.5, 5.5 or 4.0.

The relaxation behaviour and drug release profile of the L100-C-SPIONs and L100-55-C-SPIONs show similar trends (Figure 3-32C-F). Photographs (Figure 3-33) clearly showed that L100-C-SPIONs and L100-C-55-SPIONs generate clear solutions after 3 h at pH above 6.0; at pH below 6.0, L100-C-SPIONs is insoluble while L100-C-55-SPIONs generate homogenous brown suspensions. Therefore, the L100-C-SPIONs and L100-55-SPIONs showed greatest relaxivity at pH 7.4. When the pH is below pH 6.0, the relaxivity remains very low. A burst release of carmofur also can be observed at pH 7.4, and at low pHs, drug can be freed into buffers as a result of drug diffusion and Eudragit swelling.

All the values of L100-C-SPIONs and L100-55-SPIONs measured at pH 7.4 are higher than at pH 6.5. Eudragits are amphiphilic copolymers that can form micellar structures composed of a hydrophobic core and a shell with ionized carboxylate units after dissolution [41]. The pH-responsive relaxation behaviour is thought to arise because the hydrodynamic diameter of the Eudragit L100 and L100-55 micelles formed at pH 7.4 is
smaller than at pH 6.5 (Table 3-3), facilitating proton-nanoparticle interactions and thus promoting transverse relaxation [46]. The S100-C-SPIONs microparticles displayed a similar profile to L100-C-SPIONs at pH 7.4 but the $r_2$ values are slightly smaller, which again can be attributed to the hydrodynamic size of the micelles formed in the suspension. The S100 microparticles are insoluble at pH 6.5 and thus the $r_2$ value is very low: the SPIONs are encapsulated, and this prevents them from effectively interacting with the protons in solution.

Even though L100-55 is supposed to be soluble around pH 5.5, the DLS results showed that micellar or large structures formed in buffers have very large hydrodynamic size due to its larger molecular weight compared to Eudragit L and S100. As shown in Figure 3-33, the suspensions of Eudragit L100-55 in pH 5.5 is semi-transparent, which might be due to the large micellar structures. Since relaxivity is highly dependent on the distance between water protons and paramagnetic centres, the large size aggregated in suspensions inhibit the interaction between protons and SPIONs, therefore leading to very low relaxivity values.

Overall, the electrosprayed microparticles loaded with iron oxide nanoparticles showed a pH responsive relaxivity profile. However, in acidic conditions, relaxivity remained very low despite carmofur release being observed. This is because carmofur is a small molecule drug existing in the amorphous state inside the formulations, and hence can easily reach the dissolution medium through diffusion or swelling of the polymer and the permeation of water into the centre of the microparticles.

**Electrospun Fibres**

Similar experiments were performed with the fibre formulations. Figure 3-34A displays the transverse relaxivity ($r_2$) profiles. pH-responsiveness is clearly visible.
For L100-C-SPIONs-F, the transverse relaxivity increased rapidly at pH 7.4, with $r_2$ rising from $29.3 \pm 8.3$ to $66.9 \pm 2.7$ mM$^{-1}$s$^{-1}$ over 40 min and subsequently reaching $69.8 \pm 2.5$ mM$^{-1}$s$^{-1}$ at 3 h. At pH 6.5, the relaxivity increased from $13.5 \pm 2.0$ mM$^{-1}$s$^{-1}$ after 10 min immersion to $42.1 \pm 3.0$ mM$^{-1}$s$^{-1}$ after 3 h. All the values measured at pH 7.4 are higher than at pH 6.5. L100-C-SPION-F fibres had dissolved at both pHs after 3 h of immersion and the resultant solutions were clear, while the S100-based fibres are insoluble at pH 6.5 (Figure 3-35). The relaxivity profiles are consistent with those of microparticle formulations. Figure 3-34B gives the corresponding drug release, both fibres displayed fast carmofur release profiles, and the S100-C-SPIONs-F at pH 6.5 is slightly slower than that at pH 7.4, which agrees with the dissolution tests.

Figure 3-35 Photographs of the fibres in PBS buffer at different pH after 3 hour immersion, as labelled. L100-C-SPIONs-F dissolve in PBS at both pH 6.5 or 7.4, while S100-C-SPIONs-F dissolves at pH 7.4 but not pH 6.5.
3.4.5.3 \( r_2 \)-Cumulative Drug Release Fitting

To explore the relationship between carmofur release and relaxivity change, the cumulative carmofur release was plotted against the corresponding \( r_2 \) values.

**Electrosprayed Microparticles**

For electrosprayed microparticles, the plots are given in Figures 3-36 to 3-38. Generally, a good linear correlation between drug release and relaxivity can be observed in the plots with S100-C-SPIONs and L100-C-SPIONs at pH 7.4 (Figure 3-36A and 3-37A) with \( R^2 \) values of 0.9034 and 0.9559, respectively. However, \( r_2 \) did not significantly change over 3 hours at the other pH values, and therefore no clear linear trend between relaxivity and drug release profile was observed with \( R^2 \) values in a range of -0.49 to 0.91.

![Figure 3-36](image)

**Figure 3-36** The relationship between transverse relaxivity and the percentage of drug released for S100-C-SPIONs at pH 7.4 (A), 6.5 (B), 5.5 (C) and 4.0 (D).
Figure 3-37 The relationship between transverse relaxivity and the percentage of drug released for the L100-C-SPIONs at pH 7.4 (A), 6.5 (B), 5.5 (C) and 4.0 (D).

Figure 3-38 The relationship between transverse relaxivity and the percentage of drug released for the L100-55-C-SPIONs at pH 7.4 (A), 6.5 (B), 5.5 (C) and 4.0 (D).
Electrospun Fibres

For the electropsun fibres, clear linear correlations between relaxivity and drug release can be observed in all conditions (Figure 3-39) except for S100-C-SPIONs-F at pH 6.5, with $R^2$ ranging from 0.94 to 0.99. Based on this, it appears that drug release from the S100-C-SPIONs and L100-C-SPIONs microparticles at pH 7.4, S100-C-SPIONs-F at pH 7.4 and L100-C-SPIONs-F at pH 6.5 and 7.4 could be quantified in situ via relaxivity. This might be particularly helpful in treatments using highly toxic chemotherapy in environments such as the small intestine and colon, where pH lies around 6.0-7.0. Both the S100 and L100 fibres and microparticles exhibit linear relationships between drug release and $r_2$ around neutral pH values, according to the plots built on release data beyond 60 % of total carmofur (Figure 3-37A and 3-38A). The data (< 60 % of total amount) is not involved in the plots because the release is too fast to be quantified by relaxivity in the case of the particles. In contrast, with the fibres we can obtain information over the whole carmofur release profile. Additionally, the fibres possess a slower release profile, and less release under acidic conditions. This makes the fibres a better platform for targeted delivery of SPIONs and carmofur to the small intestine and colon.

Figure 3-39 The relationship between transverse relaxivity and the percentage of drug released for L100-C-SPIONs-F and S100-C-SPIONs-F at pH 7.4 or 6.5.
3.4.5.4 *Mathematical Equation Predicting Drug Release*

In the clinic, the signal intensity of MR imaging is dependent both on the relaxivity properties of the contrast agent and also on its local concentration [47]. The local concentration is related to a number of factors such as body volumes and other pathological conditions. Therefore, we normalised the $r_2$ values in each system by calculating $r_{2,t}/r_{2,\text{max}}$ to compensate for any differences caused by local concentrations of the Fe oxide NPs. The $r_{2,\text{max}}$ represents the maximum relaxivity value possible with the formulations, which manifests in our experiments as the relaxivity after 180 min, $r_{2,180}$. In the clinic, the $r_{2,t}$ can be regarded as the local MR signal intensity at a certain time point, and $r_{2,\text{max}}$ is the theoretical maximum MR signal intensity, a constant related to the specific formulation and dose.

**Electrosprayed Microparticles**

Figure 3-40 shows that plots of carmofur release from S100-C-SPIONs at and L100-C-SPIONs at pH 7.4 vs $r_{2,t}/r_{2,180}$ reveal direct proportionality, and the fitting equations are given in Table 3-5.

![Graph showing the relationship between relaxation behaviour and cumulative carmofur release](image_url)

Figure 3-40 The relationship between relaxation behaviour ($r_{2,t}/r_{2,180}$) and cumulative carmofur release of S100-C-SPIONs and L100-C-SPIONs at pH 7.4.
Electrospun Fibres

For the electrospun fibres, the results (Figure 3-41) also reveal direct proportionality between carmofur release and \( \frac{r_{2, t}}{r_{2, 180}} \) for S100-C-SPIONs-F at pH 7.4, and L100-C-SPIONs-F at pH 7.4 and 6.5. The corresponding fitting equations are also given in Table 3-7.

![Figure 3-41 The relationship between relaxation behaviour (\( \frac{r_{2, t}}{r_{2, 180}} \)) and cumulative carmofur release of S100-C-SPIONs-F and L100-C-SPIONs-F.](image)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>pH</th>
<th>Fitting equation</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100-C-SPIONs</td>
<td>7.4</td>
<td>( Drug \ release (%) = 39.4 \frac{r_{2, t}}{r_{2, 180}} + 55.1 )</td>
<td>0.88</td>
</tr>
<tr>
<td>L100-C-SPIONs</td>
<td>7.4</td>
<td>( Drug \ release (%) = 46.7 \frac{r_{2, t}}{r_{2, 180}} + 50.8 )</td>
<td>0.96</td>
</tr>
<tr>
<td>S100-C-SPIONs-F</td>
<td>7.4</td>
<td>( Drug \ release (%) = 105.8 \frac{r_{2, t}}{r_{2, 180}} + 12.4 )</td>
<td>0.97</td>
</tr>
<tr>
<td>L100-C-SPIONs-F</td>
<td>7.4</td>
<td>( Drug \ release (%) = 101.5 \frac{r_{2, t}}{r_{2, 180}} - 8.9 )</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>( Drug \ release (%) = 97.3 \frac{r_{2, t}}{r_{2, 180}} + 1.6 )</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 3-5 Mathematical relationships between cumulative drug release and relaxation profiles (\( \frac{r_{2, t}}{r_{2, 180}} \)).
3.4.5.5 Equation Validation

To further validate the predictive ability of the $r^2$ data, the equations correlating carmofur release with relaxation behaviour were applied to predict carmofur release in a new series of experiments. Relaxation behaviour changes were determined and used to predict the extent of carmofur release. The latter was then measured by UV-vis spectroscopy.

**Drug Release Prediction**

*Electrosprayed microparticles*

The results for the microparticles are presented in **Figure 3-42**. For both microparticles, the predicted carmofur release curves show similar trends to the experimental data.

![Graph](image)

**Figure 3-42** Plots of experimental (black) and predicted (red) carmofur release from S100-C-SPIONs and L100-C-SPIONs at pH 7.4.
For the fibres (Figure 3-43), the predicted drug release curves are very similar to the experimental data, indicating the potency of our theranostic approach.

Figure 3-43 Plots of experimental (black) and predicted (red) carmofur release from L100-C-SPIONs-F and L100-C-SPIONs-F fibres. Experiments were performed in pH 7.4 or 6.5 buffers at different concentrations (concentration 1, 2 and 3: 2, 1 and 0.5 mg/mL, respectively).
Data Comparison

Two fit factors $F_1$ (the difference factor) and $F_2$ (the similarity factor) (Equation 2-6 and 2-7) were applied to statistically compare the experimental dissolution profile and that calculated based on relaxivity (see in Section 2.6.2) [48]. The results are displayed in Table 3-6.

For the microparticles, all the $F_1$ and $F_2$ values lie in the range of ‘equivalent’, suggesting the reliability of our predicted curves. For the nanofibres, the $F_1$ values of L100-C-SPIONs-F at pH 6.5 and S100-C-SPIONs-F at pH 7.4 lie in the range of “equivalent”, while for the composite L100 fibres at pH 7.4 most of the $F_1$ values are also consistent with equivalent release patterns. In terms of the similarity factor $F_2$, the majority of the values are greater than 50. All these results suggest the reliability of our predicted curves.

Table 3-6 $F_1$ (difference factor) and $F_2$ (similarity factor) values comparing the predicted vs experimental release plots.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>$F_1$</th>
<th>$F_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L100-C-SPIONs pH 7.4</td>
<td>2 mg/mL</td>
<td>3.74</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL</td>
<td>6.09</td>
<td>57.9</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/mL</td>
<td>3.63</td>
<td>70.1</td>
</tr>
<tr>
<td>S100-C-SPIONs pH 7.4</td>
<td>2 mg/mL</td>
<td>2.51</td>
<td>76.1</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL</td>
<td>4.15</td>
<td>65.7</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/mL</td>
<td>5.19</td>
<td>63.6</td>
</tr>
<tr>
<td>L100-C-SPIONs-F pH 7.4</td>
<td>2 mg/mL</td>
<td>8.71</td>
<td>47.6</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL</td>
<td>16.1</td>
<td>59.6</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/mL</td>
<td>8.58</td>
<td>48.3</td>
</tr>
<tr>
<td>L100-C-SPIONs-F pH 6.5</td>
<td>2 mg/mL</td>
<td>7.49</td>
<td>59.6</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL</td>
<td>9.54</td>
<td>52.8</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/mL</td>
<td>6.48</td>
<td>61.6</td>
</tr>
<tr>
<td>S100-C-SPIONs-F pH 7.4</td>
<td>2 mg/mL</td>
<td>14.1</td>
<td>58.1</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL</td>
<td>7.69</td>
<td>41.2</td>
</tr>
<tr>
<td></td>
<td>0.5 mg/mL</td>
<td>10.9</td>
<td>56.2</td>
</tr>
</tbody>
</table>
3.4.6 Discussion

EHDA is an attractive technology that has been applied to the encapsulation of magnetic nanoparticles for pharmaceutical applications. Microspheres fabricated in previous studies were reported to have good performance in MRI contrast, remote drug release control, and to be able to induce magnetic hyperthermia for cancer treatment [49-51]. In contrast to the literature, our formulations focused on the oral delivery of colon-targeted theranostics. Here it is important to take the stability of magnetite nanoparticles in the GI tract into consideration. SPIONs are known to be stable in a neutral environment (e.g. the colon) but are prone to degrade in any acidic environments they might encounter, such as the gastric fluids [40]. Thus we used three pH-responsive polymers, Eudragit S100, L100 and L100-55, which provided excellent protection to the magnetite nanoparticles in acidic conditions.

Although magnetite nanoparticles in microspheres have widely been applied as theranostics in imaging-guided therapy [52, 53], the relationship between proton relaxation behaviour and drug release remains largely unclear. After delivery, the structure of theranostics continuously releasing drugs will undoubtedly change. Therefore, here we explore both the proton relaxivity changes and the drug release from our formulations as a function of time. Composites loaded with magnetite nanoparticles prepared via EHDA showed a pH responsive relaxivity profile which is to some extent proportional to the amount of carmofur released in the pH where Eudragit is soluble. When the pH is lower than that required for polymer dissolution, the transverse relaxivity remained very low all the time, consistent with a previous report [28].

Compared to the electroprayed microparticles, the nanofibres displayed improved correlations between relaxation behaviour and drug release, which is preferred for the development of sensitive reporters to detect abnormal microenvironments in the small intestine and colon. The better performance of nanofibres might owe to the distinct micro/macro structures of fibre and microparticles. Microparticle possesses a very high surface-to-volume ratio and thus facilitate the interaction between water and inner polymer matrix, as well as drug diffusion into solutions. Hence, the speed of drug released into mediums is much quicker than the rate of SPIONs freed from microparticles. While fibre is presented as membrane-like compact blends, water access
into inner layer might take longer. As a result, the drug release from fibres was slower, which makes a better correlation between relaxivity profile and cumulative drug release. Thus, a novel quantification method to monitor drug release based on proton relaxation change has been established. Mathematical equations were developed allowing us to predict drug release, indicating that after oral administration drug release from our formulations could be monitored based on the MR signal intensity. This offers the exciting possibility to non-invasively monitor the extent of drug release in situ. As most chemotherapeutic agents are cytotoxic and non-specific, their safety remains a critical issue and their administration should be carefully controlled and monitored in the clinic. Our theranostics could enable image-guided therapy to control and monitor drug release in such systems, providing a safer and more precise drug delivery approach [8, 54].

A number of platforms, including inorganic, polymeric or biological systems, have been used to develop theranostics [8, 45]. However, for many of these systems, their synthetic process is complicated and time-consuming, making further development and translation to industry challenging. Our theranostic systems, in contrast, are generated via EHDA in one step, removing many of these barriers. EHDA offers multiple advantages over other techniques for making polymer/drug composites, involving no use of elevated temperatures, no separate drying step, and often yielding products with high mono-dispersity through simple and accurate control of the processing parameters [55]. EHDA is a one-step process, fast, relatively cheap, and provides the opportunity for scale up to bulk production [56].

### 3.4.7 Conclusions

This work reports a new series of pH-responsive theranostic formulations based on Eudragit microparticles and nanofibres prepared by EDHA. Formulations comprising SPIONs embedded in amorphous carmofur/polymer matrices were generated. Both types of formulations are stable in acidic conditions and can protect embedded SPIONs from digestion. *In vitro* drug release studies reveal rapid release of carmofur at the pH values typical of the small intestine and colon. The MRI contrast agent behaviour (relaxivity) was monitored and shown to be pH responsive between pH 4.0 to 7.4. Investigation of the microparticles and fibres’ relaxivity behaviour in vitro showed them to have pH responsive profiles closely correlated to the extent of drug release around
neutral pHs. Based on these linear correlations, equations were developed to quantify drug release based on relaxivity. These were subsequently used to predict the carmofur release profiles in a new series of experiments, and found to be able to do so with a high degree of fidelity. The formulations developed in this work therefore have the potential to allow non-invasive monitoring of drug release in vivo via changes in MRI signal intensity, and potentially open up a route to dramatically decrease off-target side effects in chemotherapy.

3.5 References


Chapter 3

pH-Responsive nanocomposite delivery systems

Chapter 4

Dual responsive systems
4.1. Introduction

4.1.1. Stimuli in Cancer Treatment

Compared to healthy tissues, a lowered pH is often exhibited in solid tumours, together with a higher temperature and redox potential. Some enzyme systems are also overexpressed in the tumour cells [1]. These conditions are collectively referred to as the tumour microenvironment [1]. All these parameters that differ from healthy tissues can be regarded as “intrinsic stimuli”. In addition, some external triggers can be applied in the area of interest, for example local heat induced by magnetic hyperthermia or near infrared (NIR) light. These can be regarded as “extrinsic stimuli.” A summary of the range of intrinsic and extrinsic stimuli is illustrated in Figure 4-1.

![Figure 4-1](image_url)

Figure 4-1 Schematic showing the range of stimuli-sensitive nanomaterials. Reproduced with permission [2]. Copyright 2014, Elsevier.

The local release of cargo from smart stimuli-responsive systems can be triggered by these internal and external stimuli via the composition or structure of the delivery vehicle undergoing a change in physicochemical properties. A variety of polymers, biomacromolecules or inorganic materials are able to undergo such alternations and thus can be used to construct stimuli-responsive systems [3-7]. In cases where the active agents are encapsulated within the systems, release of cargo is usually caused by a
conformational transformation in the delivery vehicle as depicted in Figure 4-2 (i.e., cleavage of shell, ionisation of functional groups, carrier degradation) while in carrier–conjugate systems, the stimulus typically leads to cleavage of the linker between cargo and delivery vehicle.

![Figure 4-2 Schematic showing the drug release in systems where active agents are encapsulated (A) or carrier–conjugate systems (B).](image)

### 4.1.2. Core-Shell Structures in Delivery Systems

Core-shell materials have attracted increasing attention in pharmaceutical applications to enhance the therapeutic efficacy and reduce side effects of drugs [8, 9]. However, in terms of synthetic route, consistent and reproducible methods are still challenging, and low yields and time-consuming fabrication also remain issues preventing further development. Thus, facile and reproducible technologies that can produce core/shell systems are much sought after. Co-axial electrohydrodynamic atomization (EHDA) involves the application of an electric field to a co-flowing electrically conductive fluid system to generate submicron-to-micron sized core-shell particles or fibres. Hence, it has become popular in the development of advanced carriers for multiple functional
agents [10, 11]. For example, an acid-sensitive core-shell nanoparticle was prepared using co-axial electrospraying and subsequently linked with an antibody for the purpose of tumour-targeted, pH responsive, drug delivery. The results of in vitro drug release profiles and cytotoxicity tests showed potential in cancer treatment [12].

### 4.1.3. Poly(N-isopropylacrylamide)

Poly(N-isopropylacrylamide), PNIPAM, is a polymer characterized by amphiphilic chains with amide groups and propyl pendants. It is the most widely studied thermo-responsive polymer for biomedical applications. PNIPAM exhibits typical lower critical solution temperature (LCST) behaviour: when the temperature is raised to above 32 °C (the LCST), a reversible conformational change takes place from an expanded coil to compact globule [13]. Different strategies for controlled delivery of drugs via PNIPAM-based formulations are presented in Figure 4-3.

A variety of PNIPAM-based hydrogels has been applied in thermo-responsive drug delivery. Liu utilized PNIPAM and alginate to fabricate a formulation that displayed gel formation around the physiological temperature and could be applied as a drug delivery vehicle for the chemotherapeutic doxorubicin [14]. A dual-pH and temperature responsive system made of carboxymethyl chitosan-graft-PNIPAM-glycidyl methacrylate was reported to give stimuli-triggered release of fluorouracil [15]. The release of a hydrophilic drug incorporated inside a swollen gel can be triggered by increasing diffusion when the temperature is below LCST, while in the case of a hydrophobic agent release can be promoted by a squeezing effect caused by gel collapse upon heating.

Other than hydrogels, PNIPAM has also been grafted or coated as a shell layer onto a core comprising other vehicles such as layered double hydroxides or mesoporous silica particles (MSNs). PNIPAM functions as a dense skin preventing the release of drug loaded in the core. A PNIPAM modified MSN has been found to allow temperature-controlled release [16]. When the temperature is beyond the LCST, PNIPAM grafted on the MSNs functioned as a ‘closed gate’ that inhibited the uptake and release of fluorescein. Kawano reported gold nanorods coated with a PNIPAM hydrogel, demonstrating reversible, remote, pulsatile phase transitions and in vivo release that could be triggered by irradiation using a NIR laser [17].
Figure 4-3 Strategies to control drug release using PNIPAM-based micro/nanosystems. A: with a hydrophilic drug loaded into the swollen gel, the release of drug is triggered as a result of enhanced diffusivity when the temperature is reduced to below the LCST. B: with a more hydrophobic agent, release can be driven via a squeezing effect caused by the gel collapse when temperature is raised beyond the LCST. C: above the LCST, heterogeneous gels can form a dense skin layer, preventing incorporated drug from release. Diagram reproduced from [18]. Copyright © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

4.1.4. Co-axial EHDA

There exist obstacles to the exploration of microspheres resulting from single fluid EHDA in pharmaceutical areas. For example, active pharmaceutical ingredients in monolithic fibres or microparticles tend to yield burst release due to the high surface to volume ratio, and the extensive distribution of drug throughout the microspheres [19-21]. In addition, biological active ingredients such as bacteria or proteins must be kept in a fluid state to maintain their activities [22-25]. Therefore, co-axial EHDA has been applied to easily combine a number of ingredients including small molecules, inorganic nanoparticles, or proteins in different compartments of a core/shell formulation [26, 27].
Co-axial EDHA is a powerful tool to develop sophisticated theranostic platforms. Jin et al. reported a core/shell fibre system allowing simultaneous MRI and drug delivery for irritable bowel disease and colonic cancer. This theranostic platform comprises a pH-responsive shell and poly(ethylene oxide) core loaded with indomethacin and gadolinium-diethylenetriamine pentaacetic acid (Gd(DTPA)). The pH-responsive shell allows the active agents to be co-delivered to the colon, where PEO core will adhere to the walls of the intestine and swell to release active ingredients. Such co-delivery of therapeutic and diagnostic ingredients should make it possible to monitor disease progression during the treatment process, therefore enhancing efficacy and minimizing potential adverse effects [28].

4.2. Aims and Objectives

In this chapter, co-axial electrospray/electrospinning was employed to fabricate a dual responsive theranostic system comprising a pH responsive polymer shell loaded with SPIONs (T₂ MRI contrast agents), and a thermo-responsive PNIPAM-based core containing the chemotherapeutic carmofur. Eudragit L100 was used in the shell, since this polymer only dissolves in aqueous media with pH > 6.0.

To obtain good MRI contrast, SPIONs were loaded in the pH-sensitive sheath, where they can be released and interact with water upon dissolution of the polymer shell. Carmofur was confined to the core to minimise the risk of undesired premature drug release, which is commonly seen in monolithic formulations. PNIPAM and ethyl cellulose were used to produce a thermo responsive core compartment. Ethyl cellulose, a low-cost biocompatible polymer that is insoluble in water, was employed here with the aim of improving the electrosprayability of PNIPAM (which is known to be poor [29]) and also to promote sustained release behaviour [30]. The theranostic systems generated aimed to target delivery of a chemotherapeutic and MRI contrast agent to the small intestine and colon (Figure 4-4). Formulations were prepared with different ratios of core and shell components and loadings of active ingredients. These were fully characterized, and their functional performance assessed.
The overarching objective of this chapter was to develop a theranostic system which could potentially be applied for the local treatment of colonic cancer in response to both pH and temperature stimuli. The goals are to:

- Fabricate and characterise composite dual responsive microparticles and fibres loaded with SPIONs and carmofur.

- Explore the drug dissolution and MRI relaxation behaviour of the theranostic systems.

4.3. Experimental

4.3.1. Materials

Chemicals were purchased as follows: sodium hydroxide (Fisher Scientific Ltd); poly(N-isopropyl acrylamide) with average Mn of 40,000 Da; ethyl cellulose (EC, 48% ethoxyl, viscosity 10 cP; 5% in toluene/ethanol 80:20(lit.)); sodium chloride, N,N-
dimethylacetamide (DMAc), acetone, anhydrous ethanol, polyvinylpyrrolidone (PVP; 40 kDa), hydroxylamine hydrochloride, o-phenanthroline, xanthan gum, FeCl$_3$·6H$_2$O and FeCl$_2$·4H$_2$O (Sigma-Aldrich); Eudragit L100 (MW 12,500 Da, Röhm GmbH); and, carmofur (ChemCruz™). Ultrapure water was collected from a Millipore MilliQ system operated at 18.2 MΩ. The preparation of PVP-stabilized SPIONs are described in Section 2.1.1.

### 4.3.2. Determination of LCST

The LCST of PNIPAM was determined using a UV–Vis spectrophotometer (Cary 60 instrument, Agilent) with 1 wt% aqueous polymer solutions, by measuring transmittance at 670 nm over the temperature range of 20–60 °C. The experiments were carried out with four heating/cooling cycles using a water bath (temperature ramp of 2 °C/min) and the transmittance was recorded every 5 seconds. The LCST was identified as the temperature at which the transmittance of the sample was 50% of the maximum value.

### 4.3.3. Preparation of Solutions for EHDA

After a series of preliminary optimization experiments, co-axial EHDA was used to produce core-shell microparticles comprising a Eudragit L100 shell loaded with SPIONs and a PNIPAM/ethyl cellulose core loaded with carmofur. The recipes are shown in Table 4-1. Eudragit L100 was dissolved in a mixture of DMAc and ethanol (2:8 v/v). PNIPAM and ethyl cellulose were dissolved in a solvent system comprising DMF and ethanol (3:7 v/v).

Single fluid processing was employed to produce PNIPAM/ethyl cellulose particles or Eudragit L100 particles and fibres loaded with SPIONs and carmofur as controls. For PNIPAM-EC particles, a solution of 4% (w/v) ethyl cellulose and 4% (w/v) PNIPAM was prepared in a mixture of DMF and ethanol (3:7 v/v). For Eudragit L100 fibres and microparticles, a 12 % (w/v) or 4 % (w/v) solution of Eudragit L100 was prepared in a mixture of DMAc and ethanol (2:8 v/v). Polymer solutions were stirred vigorously for 24 h, then carmofur (to give final concentrations of 4, 8 or 12 mg/mL) and PVP-SPIONs (at final concentrations of 4, 8 or 12 mg/mL) were added to the solutions of PNIPAM/ethyl cellulose or Eudragit L100 with sonication for 20 min.
Table 4-1 Compositions of the core/shell microparticles and fibres

<table>
<thead>
<tr>
<th>ID</th>
<th>Core</th>
<th>Sheath</th>
<th>EC : PNIPAM : Carmofur : PVP-SPIONs weight ratio d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Polymer (w/v) a</td>
<td>Carmofur (w/w) b</td>
<td>Polymer (w/v) a</td>
</tr>
<tr>
<td>P-EC@EL-M1</td>
<td>2.5 % EC</td>
<td>2.5 % PNIPAM</td>
<td>23.33 %</td>
</tr>
<tr>
<td>P-EC@EL-M2</td>
<td>4 % EC</td>
<td>4 % PNIPAM</td>
<td>20 %</td>
</tr>
<tr>
<td>P-EC@EL-M3</td>
<td>6 % EC</td>
<td>6 % PNIPAM</td>
<td>18 %</td>
</tr>
<tr>
<td>P-EC@EL-F</td>
<td>8 % EC</td>
<td>8 % PNIPAM</td>
<td>18 %</td>
</tr>
</tbody>
</table>

(a: % w/v ratio of polymer to solvent; b: % w/w ratio of carmofur to core polymer; c: % w/w ratio of PVP-SPIONs to shell polymer; d: weight ratio of the different components in the final formulations)

4.3.4. Preparation of Formulations via EHDA

The detailed electrospinning process were given in Section 2.3. To produce PNIPAM-EC particles, the working voltage was fixed at 18 kV, with a collector distance of 20 cm and a flow rate of 0.8 mL/h. To produce Eudragit L100 formulations, the working voltage was fixed at 16 kV, with a collector distance of 15 cm from the needle and a flow rate of 0.8 mL/h.

For co-axial EHDA, two syringe pumps (KDS 100, Cole Parmer) were employed to control the sheath (0.7 mL/h) and the core (0.2 mL/h) flow rates. The voltage was fixed at 14 kV, with the collector placed 20 cm from the needle. A concentric spinneret comprising a small stainless steel needle (27G; outer and inner diameters of 0.42 and 0.21 mm, respectively) nested in a larger needle (18G; outer and inner diameters of 1.25 and 0.84 mm) was employed.
4.3.5. Formulation Characterization

**Morphology**

The morphology of both the microparticles and fibres were characterised using SEM and TEM, in accordance with the protocols given in Section 2.4.1

**Physicochemical Characterisation**

The microparticles and fibres were characterised using XRD, DSC, TGA and FTIR as described in Section 2.4.2

4.3.6. Stability Assay

Stability studies were performed as described in Section 3.3.4.

4.3.7. Loading Capacity and Encapsulation Efficiency

Loading capacity (LC %) and encapsulation efficiency (EE %) were measured as detailed in Section 2.5.1.

4.3.8. *In vitro* Drug Release

To mimic the conditions encountered in oral administration, drug release experiments were first performed in an acidic solution (0.1 M HCl) to simulate the gastric fluid (37 °C) for 1 hour, and then in a PBS buffer (pH 7.4) or acetate buffer (pH 5.5) at 37 °C or 25 °C. 20 mg of sample (n=3) and 10 mL of pre-heated pH 1.5 aqueous solutions were added into dialysis bags (MWCO 3500) and transferred to 30 mL of the same buffer at 37 °C. After a 1 h incubation, the dialysis bags were removed and the acidic media replaced with 10 mL of fresh PBS buffer (pH 7.4) or acetate buffer (pH 5.5) at 37 or 25 °C. The refreshed samples in dialysis bags were transferred to 30 mL of the relevant buffer at the appropriate temperature. Experiments were undertaken in a shaking incubator (100 rpm). 1 mL aliquots were withdrawn from the dissolution medium at predetermined time points and filtered through a PVDF-type syringe filter (0.22 μm). To maintain a constant volume, 1 mL of fresh pre-heated buffer was added to the dissolution vessel.
In the temperature-triggered release experiments, samples (n=6) were immersed in 5 mL of pre-heated acetate buffer (pH 5.5, 37 °C) and transferred into a dialysis bag (MW 3500), which was in turn submerged into 20 mL of the same buffer (pH 5.5) and incubated at 37 °C (100 rpm). After a 45 min incubation, half the samples (n=3) were transferred to same buffer pre-heated at 25 °C (100 rpm). 0.5 mL aliquots were periodically withdrawn from the dissolution medium and filtered through a PVDF-type syringe filter (0.22 μm). To maintain a constant volume, 0.5 mL of fresh pre-heated buffer was added to the dissolution vessel.

The [carmofur] was measured using an Agilent Cary 100 spectrophotometer, which was depicted in Section 3.3.5. Experiments were performed in triplicate and the results are reported as mean ± standard deviation (S.D.).

4.3.9. Proton Relaxivity Monitoring

Proton relaxivity ($r_{1,2}$) were measured as described in Section 2.4.3.2.

Proton Relaxivity Monitoring

To monitor changes in proton relaxivity with pH and temperature, a dispersion of approximately 16 mg of each formulation in 8 mL of a 0.1 % (w/v) aqueous xanthan gum solution was placed into a 10 mm-diameter NMR tube, which was held at 37 °C or 25 °C. The transverse relaxation time ($T_2$) was directly monitored over 24 h. The drug release was measured as described in Section 3.3.6. All experiments were performed in triplicate and the results are reported as mean ± S.D.

Drug Release Prediction

In a set of experiments to predict drug release, dispersions of fibre samples at 1 mg/mL in 0.1% w/v xanthan gum buffer (n=3) were placed into 10 mm NMR tubes. The transverse relaxation time was monitored at 37 or 25 °C up to for 24 h. At selected time points, 0.3 mL aliquots were taken from the NMR tube and the [Fe] and carmofur content in each aliquot quantified as described in Section 3.3.6.
4.4. Results

4.4.1. Characterization of PNIPAM

Cloud Point

Before fabricating the core-shell particles, the cloud point (LCST) of PNIPAM was measured using UV-vis spectrometry (with a polymer concentration of 1 wt %). PNIPAM (Figure 4-5) displayed a stable cloud point of 36 °C without any shift after three cycles of cooling and heating, showing the phase transitions to be highly reversible. Hysteresis between heating and cooling cycles can be observed in all the runs. This is because a water bath was used to control the temperature ramp (2 °C/min), and there is some time lag between the water bath temperature changing and that of the polymer solution.

![Figure 4-5 Turbidity curve of PNIPAM (1 wt%) in water during 3 repeated heating and cooling cycles. The cloud point is ~36 °C.](image)

4.4.2. Monolithic Formulations

Before producing co-axial particles, the electrosprayability of PNIPAM/ethyl cellulose (PNIPAM-EC) blends was explored. The PVP stabilized SPIONs used here were previously prepared (see in Section 2.1.1) and characterised in Section 3.4.1.
4.4.2.1. **PNIPAM/ethyl cellulose Microspheres**

**Morphology**

Carmofur and SPION loaded PNIPAM-EC particles could be prepared but have a broad size distribution and rough surfaces (Figure 4-6), with mean particle diameters of 0.75 ± 0.50 µm.

![SEM images of PNIPAM-EC microparticles](image)

Figure 4-6 SEM images (A, B and C) and size distribution (D) of blend PNIPAM-EC microparticles loaded with carmofur and PVP-SPIONs (0.75 ± 0.50 µm). Size calculated by measuring at least 100 locations in SEM using the ImageJ software.

4.4.2.2. **Eudragit L100 formulations**

The processing parameters for the shell polymer, Eudragit L100, were also optimized.

**Morphology**

Monolithic Eudragit L100 fibres and microparticles loaded with 9.7 wt% PVP-SPIONs and 9.7 wt% carmofur were obtained (Figure 4-7), with mean fibre diameter of 0.61 ± 0.16 µm and particle size of 0.38 ± 0.22 µm, respectively.
4.4.3. Core-shell Formulations

4.4.3.1. Electrosprayed Microparticles

Morphology

All three microparticles have distinct morphology from the PNIPAM-EC particles. SEM images of P-EC@EL-M1 (Figure 4-8) reveal the particles to have a concave shape with smooth surfaces and a mean size of 0.98 ± 0.40 µm. P-EC@EL-M2 also comprises concave particles, but these are more spherical in shape due to the larger w/w ratio of core polymer to shell polymer, and have sizes of 1.05 ± 0.44 µm (Figure 4-9). The SEM images of P-EC@EL-M3 depict roughly spherical particles with a diameter of 1.00 ± 0.41 µm (Figure 4-10). It is clear that morphology of the microparticles is related to the core to shell polymer w/w ratio, with an increasing proportion of core polymer leading to more spherical particles.
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Figure 4-8 SEM images (A and B), TEM image (C) and size distribution (D) of P-EC@EL-M1. Sample nomenclature as described in Table 4-1. Size calculated by measuring at least 100 locations in SEM using the ImageJ software.

Figure 4-9 SEM images (A and B), TEM image (C) and size distribution (D) of P-EC@EL-M2. Sample nomenclature as described in Table 4-1. Size calculated by measuring at least 100 locations in SEM using the ImageJ software.
TEM images showed that all three particles have two compartments. For P-EC@EL-M3, a clear dark halo can be found, consistent with a shell compartment containing PVP-SPIONs. The two compartments in P-EC@EL-M1 and 2 are overlaid, which might be due to the concave shape of the particles. The shell compartment is darker, indicating the encapsulation of SPIONs, while the core loaded with carmofur is lighter.

4.4.3.2. Electrospun fibres

Morphology

For P-EC@EL-F, SEM images (Figure 4-11) reveal linear cylindrical structures with mean diameters of 0.42 ± 0.13 μm. A small number of pores were present on the fibre surface (marked in red circles, Figure 4-11A, B), which might because of the slower evaporation rate of the core solvent. No ‘beads-on-a-string’ morphology was observed and no evidence for any particles or phase separation was present, which indicates the components of the fibres are homogeneously mixed. P-EC@EL-F also has a clear core-shell structure (Figure 4-11C, D).
Figure 4-11 SEM images (A and B, small pores marked in red circles), TEM images (C, D) and size distribution (E) of P-EC@EL-F. Sample nomenclature as described in Table 4-1. Size calculated by measuring at least 100 locations in SEM using the ImageJ software.

4.4.3.3. Solid State Properties

P-EC@EL-M1, 3 and P-EC@EL-F were selected for further study, since they have very distinct morphologies.

XRD

The XRD patterns of the formulations and raw materials are displayed in Figure 4-12. The characteristic reflections of cubic inverse spinel Fe₃O₄ can be observed in PNIPAM-EC, P-EC@EL-M1, P-EC@EL-M3 and P-EC@EL-F, indicating successful loading of PVP-SPIONs into the microparticles. The reflections at 30.2, 35.5, 43.7, 53.6, 57.1 and 62.9 ° can be readily indexed to the (022), (311), (004), (333), (115), and (044) planes of the cubic inverse spinel Fe₃O₄ (ICSD entry 77592).
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Both PNIPAM and Eudragit L100 are clearly amorphous materials, with no distinct Bragg reflections in their XRD patterns (Figure 4-12B). Carmofur is crystalline, with myriad sharp peaks present. Ethyl cellulose is semi-crystalline, combining features of both crystalline and amorphous phases. The formulations (Figure 4-12A) also show broad haloes comparable to the raw polymers (Figure 4-12B). Thus, the broad halo between 10-30° in the XRD patterns of the particles and fibres arises from the presence of amorphous polymer, which is consistent with the literature [30-32]. The characteristic reflections of carmofur are not observed in the XRD patterns of the formulations, demonstrating that it is likely present in the amorphous form owing to the very rapid drying which occurs during EHDA.

DSC

The XRD findings are supported by DSC analysis (Figure 4-13). Pure carmofur clearly exists as a crystalline material, with two sharp endothermic melting peaks visible at ca. 114 and 115 °C, consistent with the literature [33, 34]. The raw Eudragit, ethyl cellulose and PNIPAM polymers have a broad endothermic peak between 60 to 120 °C, attributed to the loss of adsorbed water. No events can be observed in the DSC profile for PVP-SPIONs. The absence of the carmofur melting endotherm in the DSC curves of the drug and PVP-SPIONs loaded formulations confirms that it is present in the form of an amorphous solid dispersion, which is consistent with the data in Section 3.4.2.2 and the literature [30-32].
Figure 4-13 DSC profiles of PNIPAM, ethyl cellulose, PVP-SPIONs, PNIPAM-EC, P-EC@EL-M1, P-EC@EL-M3, P-EC@EL-F and carmofur.

**FTIR**

Figure 4-14 displays the FTIR spectra of PNIPAM-EC, P-EC@EL-M1, P-EC@EL-M3, P-EC@EL-F and the raw materials. Raw carmofur displayed absorption bands at 1666-1720 cm\textsuperscript{-1} and at 1495 cm\textsuperscript{-1} that relate to C=O stretching vibrations. As co-polymer of methacrylic acid and methyl methacrylate, Eudragit L100 has a characteristic stretch at 1730 cm\textsuperscript{-1} that arises from C=O vibrations of esterified carboxylic groups. For ethyl cellulose, strong bands at 1046 cm\textsuperscript{-1} can be attributed to C-O stretching, while the band at 1547 cm\textsuperscript{-1} in the PNIPAM spectrum is the N-H bending of amide groups.

With the PNIPAM-EC microparticles, the characteristic stretches of ethyl cellulose, carmofur and PNIPAM can be clearly identified. For the core-shell formulations, distinctive stretches of Eudragit L100, ethyl cellulose and PNIPAM also can be seen. The C=O stretching vibrations around 1730 cm\textsuperscript{-1} become more intense in the composite formulations, as they incorporate the stretches of carmofur between 1666-1720 cm\textsuperscript{-1}. This suggests the successful incorporation of carmofur in these formulations.
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Figure 4-14 FTIR spectra of PNIPAM-EC, PNIPAM-EC, P-EC@EL-M1, P-EC@EL-M3 and raw materials.

TGA

TGA and DTA profiles (Figure 4-15) confirmed the existence of both the PVP-SPIONs and carmofur within the composite formulations. P-EC@EL-M1, M3 and P-EC@EL-F display multistage decomposition process. The small mass loss of about 3% before 100 °C can be attributed to solvent loss (e.g. ethanol, DMF, physisorbed water). Two processes at around 120 and 240 °C can be attributed to the decomposition of carmofur, and the mass loss beyond 300 °C is associated with the polymer matrix degradation. From the TGA results of the formulations, we can calculate that the iron oxide content is 12.1 % w/w for P-EC@EL-M1, 10.4 % w/w for P-EC@EL-M1, and 10.3 % w/w for P-EC@EL-F. This corresponds to closely to theoretical SPIONs loading amount (12, 10, 10 % w/w, respectively), indicating an encapsulation efficiency (EE) of ca. 100%.
Chapter 4

Dual responsive systems

From the TGA of raw materials (Figure 4-15D, E), a small proportion of residual mass can be seen even at temperatures over 500 °C (~1-2 %). Thus, the actual SPIONs LC and EE is likely to be slightly lower than calculated, which arise due to the aggregation of SPIONs during the EHDA process.

![Figure 4-15 TGA and DTA profiles of P-EC@EL-M1 (A), P-EC@EL-M3 (B), P-EC@EL-F (C), ethyl cellulose (D) and PNIPAM (E).](image)

**4.4.4. Acid Stability**

To explore the stability of our formulations in acid environments, 10 mg of each samples was immersed in 25 mL of pH 1.5 aqueous HCl, similar to the pH of gastric liquids [35]. To compare their acid stability with bare nanoparticles, 2 mg of PVP-SPIONs was subjected to the same treatment.

**4.4.4.1. Morphology**

SEM images (Figure 4-16) of the formulations following acid incubation confirm their stability under these conditions: as a result of matrix swelling, P-EC@EL-M1 becomes more spherical, while hollow structures still can be observed for the P-EC@EL-M3 microparticles. The morphology of P-EC@EL-F appears largely unaffected after exposure to the acidic conditions.
Figure 4-16 SEM images of (A) P-EC@EL-M1, (B) P-EC@EL-M3(B), and (C) P-EC@EL-F after immersion in pH 1.5 aqueous HCl for 2 h.

### 4.4.4.2. Fe Release

The [Fe] dissolved from the microparticles and nanofibres (Figure 4-17) after 2 h was below that of a control FeCl$_3$ aqueous solution ([Fe] 1 mg/L, black line), indicating less than 2.5 wt.% of the total SPION content was released from the fibres. The concentration of iron was significantly lower than the [Fe] released from bare PVP-SPIONs (between 1-10 mg/L, purple line, equating to up to 17 wt.% degradation). These results are analogous to those observed with the Eudragit microparticles and fibres in Chapter 3. Thus, the Eudragit coating in core-shell formulations can protect the SPIONs from degradation in an acidic environment, similar to monolithic Eudragit formulations.

Figure 4-17 The results of colorimetric assays to determine [Fe] in solution for PVP-SPIONs, P-EC@EL-M1, P-EC@EL-M3 and P-EC@EL-F after immersion in pH 1.5 aqueous HCl for 2 h, with two control FeCl$_3$ aqueous solutions (blue and orange, 1 and 10 mg/L respectively).
4.4.1. *In vitro* Dissolution Studies

4.4.1.1. LC and EE

The carmofur loadings of P-EC@EL-M1, P-EC@EL-M3 and P-EC@EL-F were calculated by UV-vis spectroscopy and found to be 4.9 ± 0.3 %, 7.1 ± 0.8 % and 5.1 ± 0.4 % respectively, with encapsulation efficiencies of it should be 96.6 ± 5.5, 103 ± 11, and 115 ± 6.9% (mean ± S.D., n = 3).

4.4.1.2. *In vitro* Release

To mimic the conditions encountered during oral delivery, drug release experiments were carried out at different pHs. Initially, samples were incubated at pH 1.5 (aqueous HCl solution, mimicking the gastric pH) for 1 h at 37 °C, and then subsequently transferred to pH 7.4 or 5.5 buffer (representing the pH of the intestinal tract and the tumour microenvironment, respectively). Experiments were performed at 37 or 25 °C. Although 25 °C is lower than the physiological temperature range, these experiments offer a good insight into the temperature-responsive behaviour of the formulation matrix and how it might affect the drug release.

The results of *in vitro* dissolution tests are displayed in Figure 4-18. In the first hour, over 40% of the carmofur content was freed from PNIPAM-EC, P-EC@EL-M1 and M3 into the acidic environment (Figure 4-18A-C). But only 20 % of the drug in P-EC@EL-F was released into the dissolution medium, much reduced compared to the monolithic Eudragit L100 fibres (35 %) as shown in Figure 4-18D. which is a result of Eudragit L100 being insoluble in acidic conditions and the drug embedded in the core, there is relatively little carmofur release.

The rapid release from the core@shell particles might be due to the particle structure. Though the core@shell microparticles seem to possess clear edges between the two compartments in TEM images, some of the core and shell solutions may have mixed during EHDA processing, leading to carmofur being present at the particle surface. This drug on the surface will be freed quickly into solution and generate pores on the particle surface. This effect is magnified since the core@shell particles have very high surface area to volume ratios as a result of their small size. Once pores are created, drug from further inside the particles can easily diffuse out. Pores through which drug molecules
could escape from the microparticles could also be created by swelling of the L100 shell and the permeation of water into the particle centre.

![Figure 4-18](image)

Figure 4-18 Plots showing the release of carmofur from (A) P-EC@EL-M1, (B) PNIPAM-EC, (C) P-EC@EL-M3 and (D, E) P-EC@EL-F, as measured by UV-vis spectroscopy ($\lambda_{\text{max}} = 262$ nm). Data are given from three independent experiments as mean ± S.D.

When the microparticles were transferred into a neutral PBS or pH 5.5 acetate medium there was very rapid release noted, with 80 % cumulative release reached in 3 hours. This is observed at both 25 and 37 °C, with only slightly reduced rates at the higher temperature. Release is also marginally slower at pH 5.5 than 7.4, but again this trend is not particularly marked. The particle formulations thus have only minimal ability to discriminate between different conditions of temperature and pH.

For P-EC@EL-F, a burst release can be seen in the first 4 h after transfer from the pH 1.5 buffer to PBS, reaching > 80.0 % cumulative release. Release at 37 °C is notably lower than at 25 °C. At pH 5.5, the fibres display slower release and a distinctly thermo-responsive release profile. Faster release rates and higher cumulative release
percentages are found at 25 °C. The difference in release profile at the two pHs can be attributed to the solubility of the Eudragit L100 shell: this is insoluble at pH 5.5 and thus can reduce the release of carmofur, but becomes soluble at pH > 6 resulting in rapid release when the fibres are placed in pH 7.4 PBS.

In contrast to the microparticles therefore, the core-shell fibres display thermo-responsive properties, which might because of the difference in structures. The microparticles have smaller sizes and are more easily dispersed in buffers, allowing water to effectively get access to their core via swelling of the Eudragit L100 shell. The higher surface area to volume ratios of the particles can further facilitate interactions between water and the formulations, resulting in rapid carmofur release. In contrast, the Eudragit L100 shell of the fibres can minimize release at pH 1.5, but triggers rapid release at neutral pH. This is promising for the treatment of intestinal diseases via oral administration.

### 4.4.1.3. In vitro Thermo-Responsive Release Studies

A further experiment was performed to investigate the thermo-responsiveness of the P-EC formulations, as shown in Figure 4-19.

![Figure 4-19](image)

**Figure 4-19** Plots demonstrating the release curves of (A) P-EC@EL-M1, (B) P-EC@EL-M3 and (C) P-EC@EL-F in pH 5.5 acetate buffers when the samples were first incubated at 37 °C and then either maintained at this temperature or transferred to 25 °C. Data are given from three independent experiments as mean ± S.D.

Samples (n=6) were first incubated at pH 5.5 and 37 °C for 45 min, and then half of these samples were transferred to a 25 °C water bath (stirred at 50 rpm). Carmofur release was monitored by UV-vis measurement of the supernatant at selected time points. The results further confirm the thermo-responsive release profile of P-EC@EL-F. Compared to the release curves at 37 °C, with approx. 62 % release over 180 min, faster
release was observed when the formulations were transferred to 25 °C conditions, where over 80 % of the total drug content was released. Much reduced differences were observed with the core-shell microparticles.

4.4.2. Proton Relaxation Behaviour

In order to produce theranostic platforms, SPIONs were loaded into the pH-sensitive L100 shell of our formulations for MRI purposes. To validate our hypothesis, the relaxation behaviour was investigated at different pH (5.5, 6.5 and 7.4) and temperature (25 and 37 °C). The carmofur release profile was simultaneously monitored to elucidate any relationship between the drug release and proton relaxivity.

4.4.2.1. \( r_2 \) Profile of PNIPAM-EC

![Figure 4-20 The relaxation behaviour of PNIPAM-EC at pH 7.4. Data are given from three independent experiments as mean ± S.D.](image)

Firstly, the relaxivity of the PNIPAM-EC microparticles was explored. A very low (less than 4 mM\(^{-1}\cdot s^{-1}\)) increase in relaxivity over 24 h was observed at both temperatures (Figure 4-20), with \( r_2 \) increasing from \( 0.30 \pm 0.1 \) mM\(^{-1}\cdot s^{-1}\) at 10 min to \( 2.5 \pm 1.5 \) mM\( \cdot s^{-1}\) after 24 hours at 25 °C. The low relaxivity is thought to be due to many of the SPIONs being encapsulated into insoluble EC regions of the matrix, preventing their effective magnetic interactions with diffusive water protons [36].
4.4.2.2. \( r_2 \) and Drug Release Profile of Core-Shell Materials

Figure 4-21A-C give the \( r_2 \) relaxivity profiles of the core-shell materials as a function of incubation time. Due to the very low initial relaxivity values, profiles were monitored from 10 min immersion onwards. The recovery of \( r_2 \) can be regarded as a kinetic process which is proportional to the dissolution of the Eudragit L100 shell layer [37]. For P-EC@EL-M1, it can be seen that the transverse relaxivity gradually increases over 24 hours at pH 7.4. At all timepoints studied and both temperatures, relaxivity at pH 7.4 is higher than at pH 6.5. The pH-responsive relaxation profile is analogous to those seen with the monolithic systems in Section 3.4.6, and is expected to arise from the release and potential micellisation of SPIONs during the dissolution of Eudragit L100. At pH 5.5, the relaxivity remains low over 24 hours (less than 2.2 mM\(^{-1}\cdot\text{s}^{-1}\)) because the Eudragit L100 is insoluble. The relaxation behaviours of P-EC@EL-M3 and P-EC@EL-F are similar to that of P-EC@EL-M1.
Figure 4-21 The relaxation behaviour of (A) P-EC@EL-M1, (B) P-EC@EL-M3, (C) P-EC@EL-F. Data are given from three independent experiments as mean ± S.D.
As shown in Figure 4-22A-C, the transverse relaxivity of all three formulations is heavily influenced by pH, but much less so by temperature. When the pH is above 5.5, the $r_2$ value consistently increased over 3 hours (Figure 4-21A-C).

In order to directly compare with the relaxivity data, carmofur release was quantified by UV-vis spectroscopy alongside relaxivity changes (Figure 4-23A-C). The presence of xanthan gum causes the release milieu to have a gel-like consistency, which makes it impossible to transfer between different pH media; thus, experiments were performed only at pH 5.5, 6.5 or 7.4, with no initial acid stage. All the systems displayed rapid release of carmofur over 3 hours, consistent with the in vitro drug release tests performed without xanthan (Figure 4-18).
Figure 4-23 The drug release profiles of (A) P-EC@EL-M1, (B) P-EC@EL-M3, (C) P-EC@EL-F. Data are given from three independent experiments as mean ± S.D.
4.4.2.3. \( r_2 \)-Cumulative Drug Release Fitting

To explore the relationship between carmofur release and relaxivity, plots of cumulative carmofur release vs \( r_2 \) were constructed. At pH 5.5, it can be found that while some drug release can occur via carmofur in the core reaching the dissolution medium through diffusion or swelling of the polymer and the permeation of water into the centre of the polymer matrix, the larger SPIONs cannot exit the formulations via the same route, leading to very low \( r_2 \) values over 24 h. Thus, only data at pH 6.5 and 7.4 were analysed and the results are given in Figure 4-24. These reveal a clear linear correlation between the two parameters, with \( R^2 \) ranging from 0.92 to 0.99 except for P-EC@EL-F at pH 6.5, 37 °C. This indicates that changes in \( r_2 \) signal directly correspond to carmofur release, meaning that MRI could be exploited as a non-invasive means of monitoring \textit{in situ} drug release in environments such as the small intestine and colon. This approach could provide a non-invasive route to quantification of drug release at a site of interest, and could prove particularly helpful in treatments using highly toxic chemotherapy.
Figure 4-24 Plots showing the relationship between $r_2$ value and the carmofur release data from P-EC@EL-M1 at pH 7.4, 37 °C (A), pH 7.4, 25 °C (B), pH 6.5, 37 °C (C) or pH 6.5, 25 °C (D); P-EC@EL-M3 at pH 7.4, 37 °C (E), pH 7.4, 25 °C (F), pH 6.5, 37 °C (G) or pH 6.5, 25 °C (H); P-EC@EL-F at pH 7.4, 37 °C (I), pH 7.4, 25 °C (J), pH 6.5, 37 °C (K) or pH 6.5, 25 °C (L).
Mathematical Equation

To further validate the predictive ability of the $r_2$ data, equations correlating carmofur release (%) with relaxation behaviour of P-EC@EL-F (Figure 4-25 and Table 4-2) were established as examples to predict carmofur release in a new series of experiments.

![Figure 4-25](image)

**Figure 4-25** The correlation between cumulative release (%) and $r_2 - r_{2,24}$ of P-EC@EL-F at (A) pH 7.4, 37 °C; (B) pH 7.4, 25 °C; and (C) pH 6.5, 25 °C.

**Table 4-2** Mathematical relationships between cumulative drug release (DR) and relaxation profiles ($r_2 - r_{2,24}$) for P-EC@EL-F.

<table>
<thead>
<tr>
<th>Temperature(°C)</th>
<th>pH 7.4</th>
<th>Fitting equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td></td>
<td>$DR (%) = 89.4 \frac{r_{2,t}}{r_{2,24}} - 0.74$</td>
<td>0.9904</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td>$DR (%) = 80.3 \frac{r_{2,t}}{r_{2,24}} - 3.1$</td>
<td>0.9683</td>
</tr>
<tr>
<td>25</td>
<td>6.5</td>
<td>$DR (%) = 116.6 \frac{r_{2,t}}{r_{2,24}} - 19.3$</td>
<td>0.9526</td>
</tr>
</tbody>
</table>

Drug Release Prediction

In these, relaxation behaviour changes were determined for P-EC@EL-F (n = 3) and used to predict the extent of carmofur release. The latter was then quantified by UV-vis spectroscopy and compared with $r_2$ predictions. The $r_{2,max}$ is the maximum relaxivity value possible with the formulation, which manifests in our experiments as the relaxivity after 24 h, $r_{2,24}$. In the predictive equations we use $r_{2,t}/r_{2,24}$ instead of $r_2$ because the MRI signal intensity is related both to the relaxivity properties of the CA and also to its local concentration. In the clinic, the local CA concentration might differ as a result of varied dosages, body volumes or other pathological conditions. Hence, the $r_2$ values in each system were normalised by calculating $r_{2,t}/r_{2,max}$. Plots comparing the predicted...
and observed release profiles are presented in Figure 4-26. It is clear that the predicted drug release curves based on $r_2$ are very similar to the data obtained by UV-vis spectroscopy, indicating the potency of the theranostic approach.

![Figure 4-26](image)

**Figure 4-26** Plots of experimental (black) and predicted (red) carmofur release from P-EC@EL-F at (A) pH 6.5, 25 °C; (B) pH 7.4, 25 °C; or (C) pH 7.4, 37 °C. Experiments were performed in PBS buffer at 2 mg/mL.

**Data Comparison**

Two fit factors $F_1$ (the difference factor) and $F_2$ (the similarity factor) (see Section 2.6.2) were applied to statistically compare the experimental dissolution profile acquired by UV-vis and that calculated based on relaxivity [38]. Two dissolution profiles are regarded to be “same” or “equivalent” when $F_1$ is less than 15 and $F_2$ is greater than 50 [39]. Here we use the experimental release data obtained by UV-vis spectroscopy as the reference ($R_t$), and the predicted data as the test ($T_t$). The results are shown in Table 4-
3. The $F_1$ and $F_2$ values of P-EC@EL-F all lie in the range of “equivalent”, suggesting the reliability of our predicted curves.

### Table 4-3 $F_1$ (difference factor) and $F_2$ (similarity factor) values comparing the predicted vs experimental release plots for P-EC@EL-F.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$F_1$</th>
<th>$F_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.5, 25 °C</td>
<td>10.6</td>
<td>54.4</td>
</tr>
<tr>
<td>pH 7.4, 25 °C</td>
<td>9.2</td>
<td>58.1</td>
</tr>
<tr>
<td>pH 7.4, 37 °C</td>
<td>14.1</td>
<td>51.2</td>
</tr>
</tbody>
</table>

### 4.5. Discussion

Recently, much progress has been made in stimuli-responsiveness for cancer theranostics. A number of systems have been engineered with physicochemical transformations in response to external stimuli, such as ultrasound, heat, light and magnetic field, as well as internal stimuli, including pH, redox potential, hypoxia and enzyme, etc [2]. Most interest in this field has been focused on nano-scale platforms. Nano-scale carriers could provide a versatile platform for co-delivery of a wide range of imaging and therapeutic agents and display a wide range of benefits such as tumour-targeting, or vehicle tracking in situ [40, 41]. However, it should be noticed that the major route for delivering nanoparticles to the organs and tissues is intravenous administration. Many nanoparticles often have potential risks of long-term toxicity [42-44], and tend to suffer from low bioavailability [45]. In contrast, biodegradable materials are capable of degrading into small molecular species and can be readily excreted after injection [46]. Theranostic systems able to avoid the disadvantages of intravenous administration, biodegrade, and respond to stimuli are much sought after.

In this work, we developed dual-responsive systems that could be taken orally. The encapsulated imaging and therapeutic agents are stable in the stomach pH. The pH-responsive capacity of the core-shell fibres in particular enables deliver the majority of SPIONs and carmofur to target intestinal and colorectal areas. Similar to the formulations discussed in Chapter 3, $r_2$ values rapidly increased as a result of Eudragit
dissolution, with all the formulations exhibiting good $T_2$ contrast. However, the relaxivity of the -shell formulations ($r_2 \sim 20-40$ mM$^{-1}$·s$^{-1}$) is lower than that of monolithic systems from Chapter 3 ($r_2 \sim 30-80$ mM$^{-1}$·s$^{-1}$) over 3 h. This might be because some of the SPIONs are incorporated into the insoluble EC matrix during spinning, as observed in TEM images. The fibres we developed are also found to display thermal sensitivity in both release profile and relaxation behaviour. This could be used to not only provide release information, but also report local temperature, which could be beneficial in thermal treatment of cancer.

4.6. Conclusions

In this chapter, two core@shell microparticles and a core@shell fibre were fabricated via electrohydrodynamic methods and fully characterized. All the formulations have pH responsive polymer shells loaded with SPIONs, and a thermo-responsive core with an amorphous dispersion of a model chemotherapeutic drug, carmofur. Electron microscopy images showed that microparticle shape changes with the w/w ratio of core to shell polymer, from a concave shape with a low core polymer mass content to a spherical shape with greater weight percentage of core polymer. The fibres generally have regular core-shell morphology. The encapsulation of SPIONs in the shell polymer can prevent them from digestion in acidic conditions such as gastric fluids. In vitro drug release studies reveal that the core-shell fibres displayed a pH- and thermo-responsive release profile. In contrast, the microparticles do not show notable stimuli-responsiveness. This difference is attributed to the different surface area to volume ratios of the two types of formulation. The MRI contrast agent behaviour (relaxivity) was monitored and showed a pH responsive profile between pH 5.5 to 7.4. The relaxivity of suspensions of the formulations could be used to predict the extent of drug release. The systems developed in this work thus have the potential to allow non-invasive monitoring of drug release in vivo via changes in MRI signal intensity.

In this work, we demonstrated that LCST polymers have potential in developing thermo-responsive theranostics. In the next chapter, we will focus on the application of UCST polymers in anti-cancer theranostics.
4.7. References


Chapter 5

Thermo-responsive nano-in-micro particles
5.1. Introduction

5.1.1. Thermo-Responsive Drug Delivery Systems Based on Polymers with Upper Critical Solution Temperature

Thermo-responsive polymers have been explored to provide spatially and temporally controlled delivery of anti-cancer agents. A variety of thermo-responsive systems based on lower critical solution temperature (LCST) polymers have been reported and displayed advantages including reducing dosage and side effects in vivo [1-3]. However, upper critical solution temperature (UCST) polymers have been less widely investigated. Compared to the LCST systems, they are more efficient in response to temperature because the rate of internal drug diffusion increases at higher temperature.

Copolymers of acrylamide and acrylonitrile exhibit a UCST at physiological temperatures in aqueous solutions [4-6]. Poly(acrylamide) (PAAm) is believed to have UCST behaviour because of the H-donor (−NH$_2$) and H acceptor (−C=O) units it carries, where strong intra and intermolecular hydrogen bonding can take place. However, the amide groups are ionized at the acidic environment and these charged groups can interact with water. This means that solvation of the polymer chains is favoured in water and the UCST is below the physiological temperature range [7-9]. A strategy to increase the UCST is to enhance the intra and intermolecular hydrogen bonding via introduction of more stable H-donors such as acrylonitrile [10, 11]. The corresponding cloud point of such copolymers was shown to be related to the monomer ratio. Exploiting this approach, Hei et. al prepared a hybrid system composed of mesoporous silica particles grafted with poly(acrylamide-co-acrylonitrile) (P(AAm-co-AN)) and loaded with DOX, which displayed a thermo-responsive release profile [12, 13].

In other work, Li et. a. reported a poly(acrylamide-co-acrylonitrile)-g-polyethylene glycol micelle with a UCST of 43 °C, showing thermally triggered release of DOX both in vitro and in vivo [4]. Similar micelles loaded with doxorubicin were incorporated into a light-responsive polypyrrole nanoparticle platform to realize photothermal-controlled pulsatile delivery and concurrent photothermal therapy, which was navigated by photoacoustic imaging [14]. More recently, poly(N-(2-hydroxypropyl) methacrylamide) has been utilized as a UCST-type scaffold because of its biocompatibility and non-
immunogenic nature. By modification of -OH groups with glycol amide via a degradable carbonate ester linkage, the polymers could be imparted with good UCST behaviour near the physiological temperature [15]. Poly(N-(2-hydroxypropyl) methacrylamide) could be used to encapsulate both a hydrophilic model protein and a hydrophobic dye into a coacervate phase below the UCST, showing sustained protein release in vivo [16].

### 5.1.2. Nano-in-Micro Strategies in Pharmaceutics

Nano-in-micro strategies, in which nano-agents are encapsulated in functional microspheres, is one route to develop delivery systems with desirable properties, and to improve bioavailability and therapeutic efficacy. For example, Wang et al. prepared nano-in-micro hydrogel capsules that can protect extracellular epidermal growth factor (EGF) from degradation and allows sustained and localized release of EGF. An in vivo mice model was employed to demonstrate that the microcapsules can significantly promote the proliferation of mesenchymal stem cells and thus facilitate tissue regeneration, and were more effective than the nanocarrier loaded with EGF [17].

There exist a range of nanoscale carriers which could be incorporated into microcapsules. Of these, layered double hydroxides (LDHs) stand out because of their 2D structure, with high specific surface area, and ion exchange properties [18, 19]. LDHs generally have low toxicity and high cellular permeability, making them attractive platforms for theranostic purposes. As drug carriers, they can improve the solubilization and dissolution of hydrophobic molecules, and protect and deliver fragile biomolecules like DNA/proteins in vivo [20, 21]. As diagnostic agents, LDHs have been developed for magnetic resonance imaging (MRI), computed tomography (CT), photoacoustic tomography or fluorescence imaging [22].

### 5.1.3. Theranostics for MRI-guided Thermal Therapy

Thermal therapies for cancer treatment have gained increasing traction in recent decades as our understanding of the mechanisms behind heat-induced cell killing has improved, and advancements in technology have permitted controlled and localized heating [23-25]. Thermal therapy is often preferred over chemotherapy because it can be localised and applied repeatedly in a minimally invasive fashion. A variety of techniques are now
available for controlled and targeted heating, including lasers [26], high intensity focused ultrasound [27], radiofrequency currents [28] or magnetic hyperthermia induced by magnetic nanoparticles [29].

MRI-guided thermal therapy is non-invasive, and capable of target identification, real-time monitoring of treatment and closed-loop feedback [30, 31]. The latter can be used to further adjust the energy deposition pattern based on MRI [32]. However, the application of high energy stimuli to cause heating can lead to severe damage of the skin or healthy tissues surrounding the treatment areas [31, 33]. In some cases, the energy source is unable to yield the necessary extent of ablation (e.g. for large volume tumour masses or deep lesions) [33, 34]. Signal intensity for imaging can also be a concern, with small lesions often being difficult to image owing to low MRI contrast [35]. Hence, theranostics with high hyperthermia efficiency and powerful contrast potency are required address these issues[36, 37]. Controlling thermal therapy at the tissue and cellular level can be realized using targeting strategies, usually by exploiting nanoscale systems [38, 39]. Different types of thermal therapy have been applied to develop MRI-guided systems, including magnetic hyperthermia, ultrasound, photo thermal therapy etc [23].

5.1.4. Combination of Thermal and Chemotherapy

Combination therapies are desirable in a number of disease settings to avoid drug resistance, lower the required dosage, and reduce adverse effects [23]. Hyperthermia can be an effective treatment for cancer, enabling an elevation of local temperature to promote other treatment such as chemotherapy [40]. For example, a magnetic targeted thermo-responsive theranostic nanoplatform was devised by Li and co-workers to combine magnetic hyperthermia (MHT) with magnetothermally-facilitated release of DOX [41]. In vivo T2-weighted MRI experiments proved that, via magnetic targeting, the formulation was accumulated at the tumour with a prolonged retention time. This reduces DOX distribution to off-target tissues including the heart and kidneys, and promotes the cellular uptake of DOX.

More recently, Manigandan et al. fabricated a αvβ3-targeted chitosan micellar nanoplatform carrying DOX-SPION complexes (Ab-CS-Dox-SPION) [42]. This material integrated chemotherapy and MHT to treat mice bearing triple-negative breast
cancer tumours. TNBC is the most lethal metastatic breast cancer, with average survival times of only 13-18 months [43]. Manigandan’s system showed effective targeting, with greater accumulation at the tumour site than non-targeted particles (as shown in $T_2$-weighted MRI), and magnetic hyperthermia therapy-chemotherapy significantly inhibited both primary tumour growth and metastasis (Figure 5-1A-D). In another study, magnetic nanocrystals were developed by Liu et al., who synthesized RGD-conjugated Mn–Zn ferrite particles (MNCs) loaded with PTX in a lipid surface layer [42]. In a breast tumour-bearing mouse model, the RGD-MNCs-PTX nanoparticles displayed high negative MRI contrast and outstanding magnetism-induced heat generation. With the aid of in vivo MRI, the formulation was seen to be effectively targeted to tumour neovascular epithelial cells. Upon reaching the target site, the local temperature increase caused by MHT subsequently led to a burst release of PTX.

Figure 5-1 (A) Photograph of TNBC tumour bearing Balb/c mice treated with chitosan (CS)-SPION, CS-Dox, CS-Dox-SPION, and Ab-CS-Dox-SPION formulations. (B) Tumour volumes over 16 days (error bars indicate the standard error of mean, n = 5). (C) $T_2$ signal intensity changes at the tumour site (p < 0.05, *significance with respect to diseases control and (D) $T_2$-weighted MR images. Dotted circles denote the region of interest. Reproduced with permission [42]. Copyright 2018, American Chemical Society. (CS: chitosan, Dox: doxorubicin; SPIONs, superparamagnetic iron oxide nanoparticles; Ab: antibody).
5.2. Aims and Objectives

In this chapter, nanocomposite particles were initially fabricated as shown in Figure 5-2. Microparticles comprising a thermo-responsive polymer (TRP) encapsulating SPIONs and LDHs were produced by spray drying. SPIONs are employed as MRI contrast agents, as in previous chapters. LDH particles were used as nanocarriers of anti-cancer agents (5-fluorouracil, 5FU or methotrexate, MTX, Figure 5-3). These microparticles are explored as thermo-responsive theranostics for MRI-guided cancer treatment.

Figure 5-2 Illustration of the fabrication and application of the thermo-responsive nano-in micro theranostic developed in this chapter.
The goals of this chapter are as below:

- Synthesize and characterize a copolymer of acrylamide and acrylonitrile, p(AAm-co-AN).
- Synthesize LDHs and load with MTX or 5FU.
- Fabricate and characterise nano-in-micro particles carrying SPIONs and drug loaded LDHs.
- Explore *in vitro* drug release and relaxation behaviour of the nano-in-micro particles.
- Explore the nano-in-micro particles for hyperthermia-aided chemotherapy on cancerous cells *in vitro*.

5.3. Experimental

5.3.1. Materials

Sodium hydroxide was purchased from Fisher Scientific Ltd. Sodium chloride, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone, anhydrous ethanol, polyvinylpyrrolidone (PVP; 40 kDa), xanthan gum, FeCl₃·6H₂O, FeCl₂·4H₂O, AlCl₃·6H₂O, MgCl₂·6H₂O, 5-fluorouracil (5FU), crystal violet, 3-mercaptopropionic acid, azodiisobutyronitrile, acrylamide and acrylonitrile were sourced from Sigma-Aldrich. Methotrexate (MTX) was obtained from Generon.

5.3.2. Synthesis of Thermo-Responsive Polymer (TRP)

Poly(acrylamide-co-acrylonitrile) (P(AAm-co-AN), TRP) was synthesised as follows. Acrylamide (2.88 g, 40.5 mmol), acrylonitrile (532 mg, 9.5 mmol), and
azobisisobutyronitrile (39.2 mg, 0.24 mmol) were dissolved into 50 mL of DMSO, then bubbled with N\textsubscript{2} for 30 min to remove the oxygen in the solution. The mixture was reacted at 60 °C for 3 h under stirring at 50 rpm. 1 mL of a solution of 3-mercaptopropionic acid (42.5 mg, 0.4 mmol) in DMSO was then added into the solution. After 1 h, the reaction was ended by cooling in an ice bath and the polymer was precipitated in methanol and dried under vacuum.

5.3.3. Determination of UCST

The UCST of the TRP was determined using a UV−Vis spectrophotometer (Cary 60 instrument, Agilent) with 1 wt% phosphate-buffered saline polymer solutions (pH 7.4), by measuring absorption at 670 nm over the temperature range 20−60 °C. The experiments were carried out with 3 heating/cooling cycles using a water bath (temperature ramp of 2 °C/min) and the transmittance was recorded every 5 seconds. The UCST was identified as the temperature at which the absorption of the sample was 50% of the maximum value.

5.3.4. Polymer Characterization

Gel permeation chromatography (GPC) was performed by Marwa Rizk (Department of Chemistry, UCL) and Dr Pratik Gurnani (School of Pharmacy, University of Nottingham) using a Shimadzu Prominence system fitted with a differential refractive index detector. The system was equipped with 2 x PL aquagel-OH columns (300 x 7.5 mm) and a PL aquagel 5 \( \mu \)m guard column. The eluent used was 0.1 M NaNO\textsubscript{3} (aq), set to a flow rate of 1 mL min\textsuperscript{-1} with the column oven at 55 °C. Polyethylene glycol standards (Agilent EasyVials) were used for calibration. Samples were prepared by dissolving the TRP in 0.1 M NaNO\textsubscript{3} (aq) and heating to 75 °C. 20 \( \mu \)L was then injected onto the column. Experimental molar mass (\( M_\text{n} \)) and dispersity (D) values were determined using the Shimadzu GPC software.

5.3.5. Preparation of 5FU or MTX Loaded Layered Double Hydroxides

5FU or MTX loaded LDHs were prepared as described in Section 2.2.2.
5.3.6. **Microparticles Prepared via Spray Drying**

Spray drying was performed using a mini spray dryer (Buchi B-290, Laboratory-Technik Ltd) with a closed loop. 1.0 g of TRP was firstly dissolved in 100 mL of 70 °C water containing free drug, LDH intercalates and/or SPIONs (Table 5-1). The spray nozzle tip diameter was 0.7 mm. The inlet air temperature was 120 °C and the outlet air temperature 80 °C. The liquid feed rate to the dryer was 20 mL/min, and the flow of drying gas approximately 5 m$^3$/h. Experiments were performed under constant process conditions. After letting the equipment cool down to below 60 °C, the dry powder was collected from the particle chamber.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Chemotherapeutic (w/w)$^a$</th>
<th>PVP-SPIONs (w/w)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-M</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TRP-LDH</td>
<td>18 % LDH</td>
<td>-</td>
</tr>
<tr>
<td>TRP-5FU</td>
<td>6 % 5FU</td>
<td>12%</td>
</tr>
<tr>
<td>TRP-MTX</td>
<td>6 % MTX</td>
<td>12%</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>18 % 5FU-LDH</td>
<td>12%</td>
</tr>
<tr>
<td>TRP-LDH-MTX</td>
<td>18 % MTX-LDH</td>
<td>12%</td>
</tr>
</tbody>
</table>

$a$: the weight ratio of chemotherapeutic to TRP; $b$: the weight ratio of PVP-SPIONs to TRP

5.3.7. **Formulation Characterization**

**Morphology**

The morphology of the microparticles was characterised using SEM and TEM, in accordance with the protocols given in Section 2.4.1

**Physicochemical Characterisation**

The microparticles were characterised using XRD, DSC, TGA and FTIR as described in Section 2.4.2
5.3.8.  \textit{In vitro} Drug Release

The measurement of loading capacity and encapsulation efficiency of the drug-loaded LDHs and drug loaded microparticles was conducted using the protocol described in Section 2.5.2-2.5.3.

For drug release, 20 mg of each formulation (n=3) was dispersed in 5 mL of a buffer (either pH 7.4/6.5 phosphate buffered saline (PBS), or pH 5.0 acetate buffer) and transferred into a dialysis bag (molecular weight cut-off = 3500 Da). The bag was submerged into 15 mL of the appropriate buffer and stirred at 30, 37 or 43 °C (50 rpm) using a magnetic stirrer. 0.3 mL aliquots were withdrawn from the dissolution medium at predetermined times. To maintain a constant volume, the same amount of fresh pre-heated buffer was added to the dissolution vessel. The filtrates were centrifuged for 15 min (13,200 rpm) to remove any SPIONs or microparticle residues and then analyzed with an Agilent Cary 100 spectrophotometer. MTX and 5FU quantifications were performed at $\lambda_{\text{max}}$ of 303 nm for MTX and 262 nm for 5FU, respectively. Dilutions were undertaken when necessary to bring concentrations into the linear range of the calibration curve. Experiments were performed in triplicate and the results are reported as mean ± standard deviation (S.D.).

5.3.9.  Relaxivity Measurement

Proton relaxivity ($r_1, 2$) was measured as described in Section 2.4.3.2. To monitor changes in proton relaxivity with pH, a dispersion of approximately 10 mg of each microsphere formulation in 8 mL of a 0.1 % (w/v) aqueous xanthan gum solution was placed into a 10 mm-diameter NMR tube, which was held at 43, 37 and 30 °C. The transverse relaxation time ($T_2$) was directly monitored over 48 h. At predetermined time points, 0.3 mL of suspension was taken from the NMR tube, diluted, and filtered through a PVDF-type syringe filter (0.22 μm). To measure the drug concentration, centrifugation (13,200 rpm for 10 min) was conducted to remove the SPIONs, and the supernatant analysed on an Agilent Cary 100 spectrophotometer. All experiments were performed in triplicate and the results are reported as mean ± S.D. In a set of experiments to predict drug release, dispersions of microparticles at 1 mg/mL in 0.1% w/v xanthan gum buffer (n=3) were placed into 10 mm NMR tubes. The transverse relaxation time was
monitored at 43 or 37 °C up to for 5 h. At selected time points, 0.3 mL aliquots were taken from the NMR tube and the drug content in each aliquot quantified as above.

**5.3.10. In vitro Cell Studies**

The detailed protocol for cell culture, the PrestoBlue™ cell viability assay and crystal violet staining are given in **Section 2.7**.

**5.3.10.1. Cell Viability**

For viability assays, A549 or Caco-2 cells were pre-grown in Biolite 96 well plates. $1 \times 10^4$ cells in 200 μL of medium were seeded into each well and cultured for 24 h. The medium was aspirated, and 200 μL of pre-heated DMEM containing various concentrations of TRP-LDH-drug or TRP-drug microparticles was added. The raw drugs 5FU and MTX were dissolved in dimethyl sulfoxide (DMSO) as controls, and stored at 4 °C before use. 5FU and MTX were then diluted using culture media to various concentrations. The final concentration of DMSO did not exceed 0.1 v/v % in the culture medium.

Hypertermia was induced by elevating the temperature of an incubator to 43 °C. After 60 min of exposure, cells were immediately moved back to an incubator at 37 °C and left there for 24 h. Cell viability was determined using the PrestoBlue™ cell viability assay, and the morphology imaged on an Evos XL Core light microscope after crystal violet staining.

In multi-hypertermia studies, 200 μL of pre-heated DMEM containing TRP-LDH-MTX (at a concentration of 50 ng/mL MTX) was added. Hyperthermal treatment was induced every 24 h by elevating the temperature of an incubator to 43 °C. After 60 min of exposure, cells were immediately moved back to an incubator at 37 °C. Cell viability was determined using the PrestoBlue™ assay.
5.4. Results

5.4.1. Thermo-Responsive Polymer

A thermo-responsive polymer (TRP) was prepared by copolymerizing acrylamide and acrylonitrile (Scheme 5-1) based on protocols in a previous study [12]. TRP was isolated as white solid.

\[
\begin{align*}
\text{AIBN, DMSO, 60 °C} & \quad \Rightarrow \quad \text{3-Mercaptopropionic acid} \\
\end{align*}
\]

Scheme 5-1 Schematic illustration of the reaction to synthesize TRP.

5.4.1.1. Polymer Characterization

Physiochemical Characterization

The FTIR spectrum of TRP (Figure 5-4A) shows the presence of amide absorptions at around 1653 cm\(^{-1}\) and 1614 cm\(^{-1}\). The characteristic peak at 2241 cm\(^{-1}\) can be identified as the stretching vibration of CN groups from acrylonitrile. The \(\text{sp}^2\) C-H bend around 980 cm\(^{-1}\) of acrylonitrile and acrylamide was disappeared after polymerization, instead, characteristic peak at 2940 cm\(^{-1}\) can be seen in TRP spectra, as an evidence of \(\text{sp}^3\) C-H stretch.

The disappearance of alkenyl hydrogens with a chemical shift (\(\delta \text{ ppm}\)) of 5.0-6.5 (marked in blue circles) in the \(^1\text{H-NMR}\) spectrum of TRP (400 MHz, DMSO-\(\text{d}_6\)) further confirms the polymerization of acrylonitrile and acrylamide (Figure 5-4B). The characteristic peaks (\(\delta \text{ ppm}\)) in the \(^1\text{H-NMR}\) spectrum of TRP are as follows: 1.2-1.9 (polymer backbone, -CH\(_2\)-), 2.0-2.8 (polymer backbone, -CH-CONH\(_2\) and -CH-CN), 6.6-7.8 (-NH\(_2\)).
Molecular Weight

Molecular weight and dispersity ($D$) of the synthesised copolymer were determined by GPC, and the number average $M_n$ and weight average $M_w$ found to be 114300 and 29700 g/mol, respectively, with $D$ of 3.84. The average $M_w$ is similar to the literature [23], but the $M_n$ and $D$ are larger, indicating the polymer synthesized here has a broader molecular weight distribution.

TGA and MDSC

Figure 5-5A displayed the TGA and DTA curve of TRP. The weight loss before 200 °C can be attributed to the solvent evaporation (H$_2$O, DMSO). Clearly TRP are stable until 200 °C. Modulated temperature DSC (MDSC) enable the separation of reversible and non-reversible (amorphous relaxation) events, which present in either the non-reversing or reversing heat flow. In the former, irreversible thermal events are observed (e.g. degradation). In contrast, a glass transition, a reversible process, would be expected in the reversing heat flow. This is observed in Figure 5-5B, where a change in baseline around 119.9 °C is seen in the reversing heat flow.
Cloud Point

The cloud point was determined by measuring the turbidity changes of polymer solutions with temperature, with the UCST defined as the temperature corresponding to 50% of the maximum absorption at 670 nm (with a polymer concentration of 1 wt%). The turbidity curves are shown in Figure 5-6. TRP has a UCST of 32.7 °C, and is shown to have reversible behaviour in response to temperature changes, without any shift in the cloud point after three cycles of cooling and heating.

Figure 5-6 Turbidity curves of TRP in pH 7.4 PBS buffer (1 wt%): (A) heating and (B) cooling cycle curves (n=3).
5.4.2. Drug Loaded LDHs

Pristine LDHs were first synthesised as described in Section 2.2.2. in Teflon-lined stainless steel autoclave [44] and then loaded with MTX or 5FU via ion exchange.

5.4.2.1. Morphology

Pristine LDHs

The morphology of the pristine of LDH particles was characterized via TEM (Figure 5-7), and the materials are found to consist of pseudo-hexagonal nanosheets.

![Figure 5-7 TEM images (A and B) of pristine LDHs.](image)

Drug Loaded LDHs

The morphologies of the LDH-drug particles were characterized using TEM and SEM analysis (Figure 5-8). LDH-MTX and LDH-5FU exist as plate-like nanoparticles in TEM. In SEM images (obtained after oven drying) plate-like structures still can be seen, though the drug-loaded LDH materials have become aggregated.
Figure 5-8 TEM and SEM images of (A&B) LDH-5FU and (C&D) LDH-MTX.

5.4.2.2. **Physicochemical Characterization**

**XRD**

**Figure 5-9A** presents the XRD patterns of pristine LDH, LDH-5FU and LDH-MTX. The reflections present can be indexed to the (003), (006) and (009) planes of LDH phases. Compared to the XRD pattern of the parent LDH, the (003) and (006) reflections are broader after MTX or 5FU intercalation and shift to lower angle. The latter arises owing to the incorporation of a larger drug ion between the layers causing them to move apart, and the increase in peak broadness is indicative of increased stacking defects [45, 46].
The drug loaded LDHs displayed expanded structures with basal spacings of 10.4 Å and 20.1 Å for LDH-5FU and LDH-MTX, greater than that of the pristine LDHs (8.2 Å). These observations are all consistent with the literature [47, 48]. No XRD reflections from the pure drug can be seen in the patterns (Figure 5-9B), suggesting no pure drug crystals are present.

**FTIR**

IR spectra are shown in Figure 5-10.
In the parent LDH, the stretching bands of both hydroxide and interlayer water molecule are found at approximately 3400 cm$^{-1}$, and a band at 1647 cm$^{-1}$ can be ascribed to the δ-bend of water. The low intensity peak at around 1450 cm$^{-1}$ can be attributed to the C=C stretching vibration of MTX in LDH-MTX, and the peak at 1610 cm$^{-1}$ corresponds to the water bending mode. Further, a pair of absorption bands at 1387 and 1558 cm$^{-1}$ may be attributed to COO$^-$ stretching vibrations of MTX. Similarly, successful intercalation of 5FU was confirmed by IR. There are a range of additional peaks characteristic of 5FU in LDH-5FU, which arise around 1672 cm$^{-1}$ (C=O and C=C stretches), 1586–1300 and 809 cm$^{-1}$ (ring stretching modes) [45], and 1216 cm$^{-1}$ (C–F vibrations) [49].

Figure 5-10 IR spectra of LDH, LDH-5FU, LDH-MTX, 5FU and MTX
5.4.3. Preparation of Thermo-Responsive Particles

Spray dying was used to fabricate thermo-responsive microspheres loaded with PVP stabilized SPIONs and LDH-5FU or LDH-MTX. The PVP-stabilized SPIONs used here were previously prepared (see Section 2.1.1) and characterised in Section 3.4.1.

5.4.3.1. Morphology

TRP-M and TRP-LDH

Firstly, blank TRP microparticles (TRP-M) and particles loaded with LDH (TRP-LDH) were prepared, as displayed in Figure 5-11 respectively. It can be observed that both sets of microparticles exist in a concave shape, with mean diameters of $1.28 \pm 0.53$ and $2.53 \pm 1.11 \, \mu m$ respectively. The encapsulation of LDH results in an increase in particle size and a more uneven surface.

TRP-MTX and TRP-5FU

The microparticles carrying MTX or 5FU and PVP-SPIONs (TRP-MTX or TRP-5FU) have similar morphology to TRP-M, exhibiting concave structures with relatively
smooth surfaces (Figure 5-12). The mean sizes of TRP-MTX and TRP-5FU are $1.67 \pm 0.65 \mu m$ and $1.56 \pm 0.64 \mu m$, close to that of TRP-M.

![Figure 5-12 SEM images and size distribution of TRP-MTX (A-C) and TRP-5FU (D-F), calculated by measuring at least 100 particles.](image)

**TRP-LDH-MTX and TRP-LDH-5FU**

As can be seen in Figure 5-13, microparticles carrying SPIONs and LDH-MTX or LDH-5FU are relatively larger than the drug-loaded and blank particles ($2.74 \pm 1.48 \mu m$ for TRP-LDH-MTX, $2.83 \pm 1.83 \mu m$ for TRP-LDH-5FU). They possess concave shapes with rough surfaces, analogous to TRP-LDH.
Figure 5-13 SEM images and size distribution of TRP-LDH-MTX (A-C) and TRP-LDH-5FU (D-F), calculated by measuring at least 100 particles.

5.4.3.2. Physiochemical Characterization

Elemental Mapping

Elemental mapping results of TRP-LDH-MTX and TRP-LDH-5FU (Figure 5-14) showed that Fe, Mg and Al are uniformly distributed in the particles.
Figure 5-14 Fe, Mg, and Al elemental mapping of (A-D) TRP-LDH-MTX and (E-H) TRP-LDH-5FU.

XRD

The XRD profiles of TRP-5FU and TRP-MTX are given in Figure 5-15A. Reflections of SPIONs are observed, and a broad peak from 15-25° is very similar to that seen in the pattern of TRP. No reflections from the raw drugs are seen in the composites. This indicates that amorphous solid dispersions have formed, as would be expected with
spray drying [50, 51]. A similar broad peak (15-25°) can be found in the XRD patterns of TRP-LDH-MTX and TRP-LDH-5FU, as a result of the presence of TRP (Figure 5-15B). Reflection of SPIONs can be found superimposed on this amorphous halo, suggesting the successful encapsulation of SPIONs. Distinctive reflections of the drug loaded LDH were not observed, likely because the LDH-drug peaks have low intensity.

Figure 5-15 XRD patterns of the TRP, SPIONs, MTX, 5FU, TRP-MTX/5FU (A), and LDH-MTX/5FU, TRP-LDH-MTX/5FU (B).

DSC

Additional evidence for the amorphous nature of MTX and 5FU in the TRP-drug formulations comes from their DSC profiles. A clear endotherm around 120 °C can be seen in the DSC profile of MTX (Figure 5-16A), as has previously been observed in the literature [52]. No such endotherm was observed in TRP-MTX (Figure 5-16B). For microparticles loaded with 5FU, the DSC profile provides little information because the melting of 5FU occurs at around 280 °C, beyond the temperature where TRP begin to decompose (Figure 5-4A). A broad endotherm between 40 to 110 °C is seen in the DSC data of all microparticles, and can be attributed to the evaporation of adsorbed or absorbed water.
Figure 5-16 DSC profiles of (A) raw materials and (B) the microparticles; (C) TGA (black) and DTA (red) curves for TRP; (D) MDSC curves of TRP (T_g: glass transition temperature, MDSC experiment: heating range 0-200 °C, 3 °c min-1, frequency 60s, Amplitude 1°C).

FTIR

FTIR spectra of the formulations are given in Figure 5-17. The CN stretching vibrations of TRP (2240 cm⁻¹) are visible in all formulations. For LDH-loaded microparticles, the band between 1504 to 1616 cm⁻¹ becomes broader, suggesting the encapsulation of drug loaded LDH. The characteristic peaks at 1245 cm⁻¹ of TRP-5FU arises from C–F vibrations of 5FU, while for TRP-MTX the small band at around 1503 cm⁻¹ can be attributed to the COO⁻ stretching vibrations of MTX.

Figure 5-17 IR spectra of the raw materials and microparticles.
5.4.4. *In vitro* Dissolution Studies

5.4.4.1. **LC and EE**

The LC and EE data are listed in Table 5-2. The LC of LDH-MTX (43.5 ± 6.2 %) is higher than that of LDH-5FU (14.8 ± 1.9 %), consistent with the literature [53, 54]. Both the TRP-drug and TRP-LDH-drug microparticles displayed high EE, reaching more than 89.8 %.

<table>
<thead>
<tr>
<th>Samples</th>
<th>LC (%)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH-MTX</td>
<td>43.5 ± 6.2</td>
<td>-</td>
</tr>
<tr>
<td>LDH-5FU</td>
<td>14.8 ± 1.9</td>
<td>-</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>1.9 ± 0.1</td>
<td>95.6 ± 4.3</td>
</tr>
<tr>
<td>TRP-LDH-MTX</td>
<td>5.5 ± 0.4</td>
<td>90.5 ± 6.8</td>
</tr>
<tr>
<td>TRP-5FU</td>
<td>4.8 ± 0.4</td>
<td>94.9 ± 8.7</td>
</tr>
<tr>
<td>TRP-MTX</td>
<td>4.6 ± 0.5</td>
<td>89.8 ± 10.4</td>
</tr>
</tbody>
</table>

5.4.4.2. *In vitro Release Profile*

Drug release from the microparticles was explored in PBS and acetate buffers, and at temperatures of 30, 37 and 43 °C. 37 °C is the standard physiological temperature. A warm environment of 43 °C was used to mimic the moderate temperature hyperthermia (42–45 °C) that has been utilized in clinical trials with minimal side effects [55, 56]. 30 °C (a temperature below the UCST of TRP is not likely to be experienced physiologically except at the body exterior (the skin has a typical temperature around 32 °C [57, 58]), but was selected to give insight into the temperature-responsive behaviour of the formulations. Three pHs, 5.0, 6.5 and 7.4 were explored. pH 6.5 was selected to mimic the slightly acidic pH of the tumour microenvironment, while pH 7.4 represents normal physiological conditions. pH 5.0 was chosen to mimic a late endosomal/lysosomal environment [59-61].

**Drug Loaded LDHs**

The release profiles of drug loaded LDHs were first explored at 37 °C. Both LDH-MTX and LDH-5FU displayed pH-responsive release, with more release at lower pH, ending
with more than 60% or 70% of drug freed from LDH-MTX or LDH-5FU, respectively (Figure 5-18).

**Figure 5-18** Plots showing the cumulative release of MTX from (A) LDH-MTX (37 °C); (B) LDH-5FU (37 °C).

**TRP-MTX/5FU**

Data for TRP-5FU and TRP-MTX are given in Figure 5-19. Significant burst release (>50% in the first hour) was seen with both the TRP–drug materials, regardless of pH and temperature. This presumably arises due to the molecular distribution of drug in the polymer carrier and the high surface area of the microparticles. Thus, even when the polymer is insoluble the drug can diffuse out of the matrix into solution. Both TRP-MTX and TRP-5FU display accelerated drug release at higher temperatures. There is no clear pH response noted, though there appears to be a slight tendency for more rapid release at lower pHs.
Figure 5-19 Plots showing the cumulative release of drug from (A) TRP-MTX (37 °C) or (C) TPR-5FU (37 °C) with an enlargement of first 7 h of TRP-MTX (B) or TRP-5FU (D).

**TRP-LDH-MTX/5FU**

The drug release profiles over 120 h from the TRP-LDH-drug composites are included in Figure 5-20, with an insert panel reflecting an enlargement of the first 7 h.
Figure 5.20 Cumulative release of 5FU from TRP-LDH-5FU in pH (A) 7.4 or (B) 6.5 PBS at different temperatures over 120 h; (C) a comparison of 5FU release at different pHs and 37 °C; cumulative release of MTX from TRP-LDH-MTX in pH (D) 7.4 or (E) 6.5 PBS at different temperature over 120 h; (F) a comparison of MTX release at different pHs and 37 °C. Data obtained by UV-vis analysis and given from three independent experiments as mean ± S.D.

At 43 °C, a burst release of up to 60% can be observed in the first 2 hours, and the TRP-LDH-drug composites display very rapid release as a result of polymer matrix
dissolution. The systems at 30 or 37 °C all present a rapid release in the initial period (0-3 h), followed by very slow release (3-120 h). The first stage of release is mainly driven by the presence of some LDH–drug particles at the surface of the microparticles [62]. The second stage of release arises due to the drug escaping from LDH particles at the inside of the polymer matrix. Compared to the TRP-drug systems, the TRP-LDH-drug composites can reduce the initial burst of drug release and demonstrate a more markedly temperature responsive profile. Further comparison of the release data of TRP-LDH-drug at different pHs (37 °C) demonstrates there is some pH-responsiveness with these formulations too, with more rapid release at lower pH values (Figure 5-20C, F). Overall, the results showed that the nano-in-micro formulations prolong drug release and significantly enhance the responsiveness to temperature.

5.4.5. Release Kinetics

The release profile is controlled by several factors: the diffusion behaviour of the replacement ions/molecules entering the polymer/LDH matrix, the ion-exchange reaction time, and the diffusion of drug ions out of the microparticles. This process is rapid at 43 °C because the polymer is hydrophilic and soluble at this temperature; thus, water quickly diffuses into the polymeric matrix. While TRP is more hydrophobic around the physiological temperature (37 °C), resulting in a slower release rate. At lower temperatures 30 °C the polymer is totally insoluble, hence this diffusion process is very slow and release extending for several days is observed.

5.4.5.1. Zero-order Model

At 37 or 30 °C, TRP microparticles give an almost constant rate of release between 24–120 h, and fitting zero-order kinetics (Equation 2-3) to the data over this period gives good fits ($R^2 = 0.94$-$0.99$, Table 5-3), as displayed in Figure 5-21 and 22 [62]. While at 43°C, the zero-order model doesn’t fit well (Figure 5-21A, and 5-22A, D). Although TRP-LDH-5FU at pH 6.5, 43 °C (Figure 5-21D) displayed a good fit, it is meaningless since release roughly doesn’t increase after 24 h.
Figure 5-21 Fits of the zero-order release equation to the data between 24 and 120 h for TRP-LDH-MTX (A-C, pH 7.4, 43, 37, 30 °C, respectively; D-F, pH 6.5, 43, 37, 30 °C, respectively; G, pH 5.0, 37 °C).
Chapter 5

Thermo-responsive nano-in-micro particles

Figure 5-22 Fits of the zero-order release equation to the data between 24 and 120 h for TRP-LDH-5FU (A-C, pH 7.4, 43, 37, 30 °C, respectively; D-F, pH 6.5, 43, 37, 30 °C, respectively; G, pH 5.0, 37 °C).
The fitting results suggest the TRP-LDH-drug composites are capable of providing sustained delivery of chemotherapeutics for cancer treatment. Hence, they provide two delivery options: fast release can be triggered to arrest rapidly growing tumours by raising the surrounding tissue temperature via the application of an external heat source, and without this stimulus the formulations act as slow release matrices to maintain the drug concentration at a therapeutic level over a prolonged period of time.

### Table 5-3 The results of fitting the zero-order model to MTX/5FU release between 24 – 120 h to the TRP-LDH-MTX/TRP-LDH-5FU formulations (see plots in Figure 5-20 and Figure 5-21).

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>R² (24-120 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-LDH-MTX</td>
<td>7.4</td>
<td>43</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.96</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>7.4</td>
<td>43</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.99</td>
</tr>
</tbody>
</table>

#### 5.4.5.2. Peppas Model

To understand better the mechanism of drug release, the Peppas model (Equation 2-2) was fitted to the first 60% of the release data [63]. The results are summarized in Figure 5-23 to 26 and Table 5-4. Generally the model fits well, although some of the fits for TRP-LDH-5FU (pH 6.5, 30 °C, pH 6.5, 37 °C and pH 5.0, 30 °C) and TRP-LDH-MTX (pH 5.0, 30 °C) are relatively poor. Release data for the TRP-drug composites commonly display poorer correlation with the Peppas model, with R² in the range of
0.75-0.97. The Peppas exponents are in the range 0.45 to 0.89, suggesting drug release occurs through a combination of matrix swelling and drug diffusion.

Figure 5-23 Fits of the Peppas model to the release data of TRP-LDH-MTX (A-C, pH 7.4, 43, 37, 30 °C, respectively; D-F, pH 6.5, 43, 37, 30 °C, respectively; G, pH 5.0, 37 °C).
Figure 5-24 Fits of the Peppas model to the release data for TRP-LDH-5FU (A-C, pH 7.4, 43, 37, 30 °C, respectively; D-F, pH 6.5, 43, 37, 30 °C, respectively; G, pH 5.0, 37 °C).
Figure 5.25 Fits of the Peppas model to the release data of TRP-MTX (A, pH 7.4, 43, 37, 30 °C, respectively; D, pH 6.5, 37 °C).

Figure 5.26 Fits of the Peppas model to the release data of TRP-5FU (A, pH 7.4, 37, 30 °C, respectively; C, pH 6.5, 37 °C).
Table 5-4 The results of fitting the Peppas model to the first 60% or first 2 h release from the TRP-drug and TRP-LDH-5FU systems, extracted from the plots in Figure 5-22 to 25.

<table>
<thead>
<tr>
<th>Fibres</th>
<th>pH</th>
<th>T (°C)</th>
<th>Fitting Data</th>
<th>Peppas model</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-MTX</td>
<td>7.4</td>
<td>43</td>
<td>First 60%</td>
<td>$Q_t = 74.5 t^{0.54}$</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td>First 60%</td>
<td>$Q_t = 65.4 t^{0.58}$</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>First 60%</td>
<td>$Q_t = 50.6 t^{0.78}$</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>37</td>
<td>First 60%</td>
<td>$Q_t = 65.6 t^{0.46}$</td>
<td>0.99</td>
</tr>
<tr>
<td>TRP-LDH-MTX</td>
<td>7.4</td>
<td>43</td>
<td>First 60%</td>
<td>$Q_t = 52.8 t^{0.47}$</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 31.0 t^{0.44}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 23.0 t^{0.60}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>First 60%</td>
<td>$Q_t = 50.6 t^{0.54}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 40.0 t^{0.47}$</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 27.1 t^{0.77}$</td>
<td>0.96</td>
</tr>
<tr>
<td>TRP-5FU</td>
<td>7.4</td>
<td>37</td>
<td>First 60%</td>
<td>$Q_t = 67.8 t^{0.38}$</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>First 60%</td>
<td>$Q_t = 60.9 t^{0.50}$</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>37</td>
<td>First 60%</td>
<td>$Q_t = 69.1 t^{0.38}$</td>
<td>0.75</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>7.4</td>
<td>43</td>
<td>First 60%</td>
<td>$Q_t = 62.3 t^{0.30}$</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 34.2 t^{0.37}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 28.1 t^{0.53}$</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>First 60%</td>
<td>$Q_t = 48.2 t^{0.54}$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 38.8 t^{0.22}$</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>0-2h</td>
<td>$Q_t = 29.2 t^{0.26}$</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>First 60%</td>
<td>$Q_t = 54.2 t^{0.46}$</td>
<td>0.87</td>
</tr>
</tbody>
</table>

### 5.4.5.3. Bhaskar Model

The Bhaskar model (see in Equation 2-4) assumes that diffusion through the particle is the rate limiting step to release, making it appropriate for ion-exchange processes.

**Fitting of Data from 0-120 h**

Figure 5-27 depicts the fits to release data (0-120 h) obtained with the Bhaskar equation: the release data are clearly nonlinear with $R^2$ typically below 0.9 (Table 5-5).
Chapter 5

Thermo-responsive nano-in-micro particles

Figure 5.27 Fits of the Bhaskar equations to the release data (0-120 h) for (A-B) TRP-LDH-MTX microparticles, and (C-D) TRP-LDH-5FU.

Table 5.5 The results of fitting the Bhaskar model to drug release (0-120 h) from TRP-LDH-MTX and TRP-LDH-5FU, extracted from the plots in Figure 5.26.

<table>
<thead>
<tr>
<th>Fibres</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-LDH-MTX</td>
<td>7.4</td>
<td>43</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.70</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>7.4</td>
<td>43</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.56</td>
</tr>
</tbody>
</table>
Since two distinct stages can be clearly seen in the release profiles, the Bhaskar model was separately fitted to the release data from 0-7 h (first stage) and 7-120 h (second stage).

**Fitting of Data from 0-7 h**

Fits of the Bhaskar model to the first 7h of release data are presented in Figure 5-28. Clearly, Bhaskar equation does not give good fit, which indicating that the diffusion of drug through the thermo-responsive matrix is the rate limiting step to release (rather than ion-exchange in the LDH particles) at the first stage (0-7h).

Figure 5-28 Fits of the Bhaskar equations to the release data (0-7 h) for (A-B) TRP-LDH-MTX microparticles, and (C-D) TRP-LDH-5FU.
Table 5-6 The results of fitting the Bhaskar model to drug release (0-7 h) from TRP-LDH-MTX and TRP-LDH-5FU, extracted from the plots in Figure 5-27.

<table>
<thead>
<tr>
<th>Fibres</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-LDH-MTX</td>
<td>7.4</td>
<td>43</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.82</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>7.4</td>
<td>43</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.58</td>
</tr>
</tbody>
</table>

**Fitting of Data from 7-120 h**

Fits of the Bhaskar model to the second stage of drug release can be found in Figure 5-29. At 30 and 37 °C, the fits are much better than for the first stage, while at 43 °C the fits are very poor (Table 5-7). This indicates that at the lower temperatures ion exchange is the rate limiting step to release.
Figure 5-29 Fits of the Bhaskar equations to the release data (7-120 h) for (A-B) TRP-LDH-MTX microparticles, and (C-D) TRP-LDH-5FU.

Table 5-7 The results of fitting the Bhaskar model to drug release (7-120 h) from the TRP-LDH-MTX and TRP-LDH-5FU particles, determined from the plots in Figure 5-28.

<table>
<thead>
<tr>
<th>Fibres</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-LDH-MTX</td>
<td>7.4</td>
<td>43</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.80</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>7.4</td>
<td>43</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>43</td>
<td>-0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>37</td>
<td>0.97</td>
</tr>
</tbody>
</table>
Summary

In total, three kinetic models (Peppas, Bhaskar and zero order models) were used to analyze the release behaviour of therapeutic agents from the TRP-LDH-drug formulations at different conditions. At 43 °C, Peppas exponents suggest that the rapid dissolution at the first stage (0-7h) of drug loaded result from combined effects of matrix swelling and drug diffusion, rather than ion-exchange in the LDH particles (based on poor fitting correlation with Bhaskar equations). Similar situations can be found at lower temperatures. Since the majority of therapeutic agents were released in the first stage (0-7h) at 43 °C as a result of matrix dissolution, the data at the following stage (7-120 h) cannot be well fitted into either the zero-order or Bhaskar model. On the contrary, both the zero-order and Bhaskar equation give good fit with 7-120 h data at 30 and 37 °C, suggesting that ion exchange is the rate limiting step in the sustained release stages. Thus, our TRP-LDH-drug complexes can serve as either quick release platforms or sustained release systems with an initial burst stage.

5.4.6. Proton Relaxivity

5.4.6.1. $r_2$ and Drug Release Profile

The $T_2$ relaxation behaviour of the TRP-LDH-drug particles was investigated at three different temperatures (30, 37 and 43 °C). The drug release profile was monitored as a function of incubation time to elucidate any relationship between the drug release and proton relaxation changes.

The relaxivity profiles of the LDH-containing microparticles as a function of incubation time are shown in Figure 5-30. The initial $r_2$ value (measured after 10 min of immersion) was low. The $r_2$ values then increased with time as the microparticles remained suspended, as shown in Figure 5-30A, C. This is due to the dynamic process of matrix dissolution/swelling as reported in [64]. The dissolution/swelling process enables water molecules to come into contact with the SPIONs, boosting diffusive water access and hence enhancing their relaxation rates and relaxivities. Thus, instead of measuring a single $r_2$ value, the relaxivity was monitored as a function of time during incubation at different temperatures. The resultant $r_2$ profiles can help to determine whether MRI
signal could be utilised as a mechanism of monitoring microparticles dissolution/swelling, and hence drug release and temperature change.

At 30 °C the $r_2$ values remained very low over 48 h (~ 1 mM$^{-1}$·s$^{-1}$). This is because the TRP is hydrophilic at this temperature, thus the MRI-active SPIONs stayed inside the formulations, preventing their effective magnetic interaction with diffusive water protons. The drug release profiles (Figure 5-30B, D) are all rapid in the first 3 hours at all temperature, as was seen in the previous dissolution tests.

5.4.6.2. Thermo-responsive Relaxivity

The overall relaxivity at 43 °C is higher than at 37 °C, and the $r_2$ vs time profile is clearly thermo-responsive. To further explore this, $r_2$ of TRP-LDH-5FU in PBS buffer (pH 7.4) was explored as a function of incubation time at a range of further temperatures (35 -
46 °C). The results are given in Figure 5-31A. $r_2$ values rapidly increased after less than 1 hour of incubation at all temperatures except 35 °C, and the profiles are clearly thermo-responsive around physiological temperatures. This can be related the variation in hydrophilicity of TRP with temperature.

![Figure 5-31](image)

Figure 5-31 (A) The $r_2$ vs time profile of TRP-LDH-5FU at different temperatures; (B) the relationship between temperature and relaxation behaviour ($r_2$) after 1 hour of incubating TRP-LDH-5FU.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Z-average (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>46.0</td>
<td>204 ± 16</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>43.0</td>
<td>232 ± 8</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>40.0</td>
<td>377 ± 8</td>
<td>0.61 ± 0.05</td>
</tr>
<tr>
<td>37.0</td>
<td>1216 ± 90</td>
<td>0.42 ± 0.19</td>
</tr>
<tr>
<td>35.0</td>
<td>2224 ± 36</td>
<td>0.24 ± 0.36</td>
</tr>
<tr>
<td>30.0</td>
<td>8876 ± 801</td>
<td>1</td>
</tr>
</tbody>
</table>

As revealed by DLS data on TRP-LDH-5FU microparticles after 2 h incubation (Table 5-8), the microparticles undergo a rapid coil-to-globule transition with a decreasing temperature around their UCST, with reduced diffusive water access meaning lessened relaxivity enhancement. The water miscibility of TRP above the UCST results in a higher surface area to volume ratio and a greater surface area for SPION-water interactions [65]. Plotting the $r_2$ values after 1 hour of incubation (where $r_2$ reaches a
plateau and becomes stable) against temperature shows a clear linear correlation, which suggests the potential of the formulations to allow in situ monitoring of the temperature by MRI (Figure 5.31B).

It can be observed that \( r_2 \) undergoes a dramatic increase in the first 30 min in the relaxivity curves between 37-46 °C (Figure 5.31A). We hypothesized that the relaxivity value over this initial period is closely dependent on both temperature and the incubation time. To test this, a binary linear regression was conducted using temperature and incubation time as independent variables, and \( r_2 \) data in the first 30 min as the dependent variable. Mathematical equations could be constructed with a \( R^2 \) of 0.90 (Figure 5.32), indicating a good linear correlation.

\[
\begin{align*}
\text{(A)} & \\
r_2 &= 24.2t + 2.5T - 95.7 \\
R^2 &= 0.90
\end{align*}
\]

Figure 5.32 (A) results of binary linear regression on \( r_2 \) data of TRP-LDH-5FU over the first 30 min vs time (t) & temperature (T), and view of (B) time-\( r_2 \) or (C) temperature-\( r_2 \) panels.

Hence, this formulation can provide a potential strategy utilizing the dynamic structural transformation of the TRP at temperatures beyond the UCST to monitor in real-time the temperature by MRI. Since high temperatures in hyperthermia can harm healthy surrounding tissues, it is desirable to temporally and spatially monitor the temperature during treatment.
5.4.6.3. $r_2$-Cumulative Drug Release Fitting

The relationship between drug release profiles and relaxivity at 43 or 37 °C was explored, and plots of cumulative drug release vs $r_2$ were constructed over 300 min. Here we normalised the $r_2$ values in each system by calculating $r_2_t / r_{2,max}$ to establish predictive equations at 43 and 37 °C. The data at 30 °C were not analysed because $r_2$ values at that pH remained low over 48 h. The $r_{2,max}$ represents the maximum relaxivity value possible with the formulations, which manifests in these experiments as the relaxivity after 48 h. In the clinic, the $r_{2,t}$ can be regarded as the local MR signal intensity at a certain time point, and $r_{2,max}$ is the theoretical maximum MR signal intensity, a constant related to the specific formulation and dose.

![Figure 5-33](image)

**Figure 5-33** Correlations between cumulative drug release and $r_{2,t}/r_{2,48}$ of TRP-LDH-MTX at (A) 43 and (B) 37 °C and TRP-LDH-5FU at (C) 43 and (D) 37 °C.

*Figure 5-33* shows that plots of drug release vs $r_{2,t}/r_{2,48}$ reveal direct proportionality ($R^2 > 0.90$), and the best fit equations are given in *Table 5-9*.
Table 5-9  Mathematical relationships between cumulative drug release (DR) and relaxation profiles ($r_2/t_2$).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>T(°C)</th>
<th>Fit equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-LDH-MTX</td>
<td>43</td>
<td>$DR (%) = 42.5 \frac{r_{2,t}}{r_{2,48}} + 25.2$</td>
<td>0.9463</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>$DR (%) = 35.3 \frac{r_{2,t}}{r_{2,48}} + 18.5$</td>
<td>0.9206</td>
</tr>
<tr>
<td>TRP-LDH-5FU</td>
<td>43</td>
<td>$DR (%) = 35.6 \frac{r_{2,t}}{r_{2,48}} + 25.6$</td>
<td>0.9558</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>$DR (%) = 23.4 \frac{r_{2,t}}{r_{2,48}} + 18.2$</td>
<td>0.9229</td>
</tr>
</tbody>
</table>

To further validate the predictive ability of the $r_2$ data, the equations correlating release with relaxation behaviour were applied to predict drug release in a new series of experiments. Relaxation behaviour changes were determined at 1 mg/mL (n=3) and used to predict the extent of drug release. The latter was then measured by UV-vis spectroscopy. Figure 5-34 gives the predicted and experimental release curves.

Figure 5-34 Plots of experimental (black) and predicted (red) drug release from: TRP-LDH-MTX at (A) 37 and (B) 43 °C; TRP-LDH-5FU at (C) 37 and (D) 43 °C. Experiments were performed in pH 7.4 PBS buffer, at 1 mg/mL (n=3).
The ‘similarity’ of the predicted and experimental curves was analysed by two fit factors $F_1$ (the difference factor) and $F_2$ (the similarity factor) (see in Section 2.6.2), which were applied to statistically compare the experimental dissolution profile and that calculated based on relaxivity [66]. Two dissolution profiles are regarded to be “same” or “equivalent” when $F_1$ is less than 15 and $F_2$ is greater than 50 [67].

### Table 5-10 $F_1$ (difference factor) and $F_2$ (similarity factor) values comparing the predicted vs experimental release plots.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$F_1$</th>
<th>$F_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-LDH-MTX 43 °C</td>
<td>8.7</td>
<td>63.3</td>
</tr>
<tr>
<td>TRP-LDH-MTX 37 °C</td>
<td>13.8</td>
<td>58.5</td>
</tr>
<tr>
<td>TRP-LDH-5FU 43 °C</td>
<td>8.2</td>
<td>63.1</td>
</tr>
<tr>
<td>TRP-LDH-5FU 37 °C</td>
<td>10.4</td>
<td>66.1</td>
</tr>
</tbody>
</table>

As detailed in Table 5-10, all the predicted values lie in the range of “equivalent”. Both microparticles at 43 °C displayed higher similarity (lower $F_1$ and higher $F_2$ ) compared to curves at 37 °C, which is consistent with the results in Figure 5-34. For both microparticles at 43 °C, the predicted and experimental curves generally are similar in shape, while at 37 °C the predicted curves are different from the experimental data. These results suggest that the fitting equations built on $r_2$ profile could be generally reliable to predict the release at 43 °C.

5.4.7. In vitro Cell Studies

Hyperthermia has been used clinically as an adjuvant to chemo- or radiotherapy [68, 69] since it can enhance local perfusion of drugs, sensitise cancer cells to cytotoxic agents, suppress DNA repair, and improve tissue oxygenation [70, 71]. However, using temperatures over 50 °C can be harmful to healthy surrounding tissues, while the damage induced by mild heating stress (42-45 °C) tends to be repaired by a cancer cell’s intrinsic thermostability system. A combination of mild hyperthermia and chemotherapy can dramatically enhance treatment efficacy. Here cell studies were conducted to explore the anti-cancer effect of our formulations, combining chemotherapy and mild hyperthermia (MHT). Given the fact that the local hyperthermia therapy is generally conducted for 20–60 min at a temperature between 42–45 °C [72-
74], cells treated with TRP-drug or TRP-LDH-drug were first incubated at 43 °C for 1 h before moving to physiological temperature (37 °C).

5.4.7.1. Biocompatibility of TRP-M

The cytotoxicity of the TRP-M microparticles to Caco-2 or A549 cells was explored at a range of concentrations after 24 h incubation, with or without hyperthermia (Figure 5-35). Cell viability were evaluated via PrestoBlue™, a cell metabolic activity assay. The cell viability at 37 °C is always high, indicating that the microparticles are biocompatible and do not induce cytotoxicity to either cell type (viability > 95 %). Viability of both cell lines decreased when MHT was conducted in isolation (p<0.05).

![Figure 5-35 In vitro cytotoxicity data for (A) Caco-2 and (B) A549 cells treated with different concentrations of TRP-M microparticles; with or without mild hyperthermia treatment (MHT) (*p<0.05). Data given from four independent experiments as mean ± S.D and compared by Student t-test. The untreated cells control is used as a baseline with a viability of 100%.

5.4.7.2. Hyperthermia-aided chemotherapy

To explore the synergistic effect of hyperthermia and chemotherapy, TRP-LDH-drug, TRP-drug or free drug in solution (at a concentration equal to 100 % release) were applied to both cells exposed to hyperthermal conditions (43 °C, 60 min) (Figures 5-36). Chemotherapy without any heat treatment induced cell death in both Caco-2 and A549 cultures treated with 5FU or MTX (Figure 5-36A, B, E, F). A significant decrease in cell viability can be seen upon heat exposure except for A549 cells treated with 5FU. A similar decrease can be found in cells treated with TRP-drug microparticles (Figure 5-36C, D, G, H).
Figure 5-36 *In vitro* cytotoxicity data for chemotherapeutic and TRP-drug microparticles: (A) Caco-2 and (B) A549 cells treated with MTX; (C) Caco-2 and (D) A549 treated with TRP-MTX; (E) Caco-2 and (F) A549 cells treated with 5FU; (G) Caco-2 and (H) A549 cells treated with TRP-5FU; with or without mild hyperthermia treatment (MHT). Data given from four independent experiments as mean ± S.D. *p<0.05, **p<0.01, data compared by Student’s t-test. The untreated cells control is used as a baseline with a viability of 100%.

Similar to the drugs and TRP-drug formulations, the TRP-LDH-5FU and TRP-LDH-MTX formulations are toxic to the cells (Figure 5-37). In most cases, the viability of
both cell lines decreased by a greater extent after exposure to high temperature (p<0.01-0.001) compared to the results found with the drugs or TRP-drug formulations with MHT. Visual observation showed a decrease in the cell numbers (Figure 5-38), confirming the anti-proliferative effect of the chemotherapy and hyperthermia combination. With the TRP-LDH-drug materials, both cell types displayed higher viability compared to those treated with only the drug, since not all the drug was released during the in vitro experiments. When combined with hyperthermia, the cell death induced by TRP-LDH-drug microparticles was significantly increased.

Figure 5-37 In vitro cytotoxicity data: (A) Caco-2 and (B) A549 cells treated with TRP-LDH-MTX; (C) Caco-2 and (D) A549 cells treated with TRP-LDH-5FU; with or without mild hyperthermia treatment (MHT). Data given from four independent experiments as mean ± S.D. An untreated cells control is used as a baseline with a viability of 100%. **p<0.01, ***p<0.001. Cell viability data were compared by Student's t tests.
Figure 5-38 Morphological changes visualized under a light microscope for: (A) Caco-2 cells and Caco-2 cells treated with (B) mild hyperthermia treatment (MHT), (C) TRP-LDH-MTX and (D) MHT+TRP-LDH-MTX; (E) A549 cells, and A549 cells treated with (F) MHT, (G) TRP-LDH-MTX and (H) MHT+TRP-LDH-MTX (Scale bar: 100 μm).

Further studies exploiting a multiple MHT strategy were performed. TRP-LDH-MTX was selected for these studies since it displayed the best anti-proliferation effects with MHT. Caco-2 and A549 cell lines were treated with TRP-LDH-MTX and exposed to high temperature (43 °C, 60 min) every 24 hours for three days. The results are given in Figure 5-39. Clearly, when the MTH treatment was repeated the cytotoxic effects of TRP-LDH-MTX are markedly enhanced, with a cell death rate greater than 80% after three MHT treatments. This presumably arises from an increased amount of MTX being freed from the microparticles with time, leading to enhanced chemotherapy aided by hyperthermia stress.

Figure 5-39 In vitro cytotoxicity data for (A) Caco-2 and (B) A549 cells treated with TRP-LDH-MTX and multiple mild hyperthermia treatment (MHT) (24, 48 and 72h, 37 °C; 1, 2, and 3 times, 43°C, 60 min, respectively). Data given from four independent experiments as mean ± S.D. An untreated cells control is used as a baseline with a viability of 100%. **p<0.01, ***p<0.001. Cell viability data were compared by Student’s t tests.
Previous studies have shown that a combination of hyperthermia and cytotoxic agents can synergistically inhibit the proliferation of Caco-2 and A549 cell lines [75-77]. In our study, we found that this synergy can be further amplified with the aid of our thermo-responsive TRP-LDH-drug formulations. The enhancement in synergistic effects can be explained by the following reasons. First, an increased amount of the chemotherapeutic was released from the TRP-LDH-drug systems at higher temperatures. Hyperthermia also promoted cell perfusion, facilitating the diffusion of 5FU or MTX into the cell membrane [78]. Finally, moderate heat treatment affects DNA repair mechanisms, promoting cell apoptosis after chemotherapy [79]. Overall, these results confirmed that our formulations can enhance the synergistic effect of hyperthermia aided chemotherapy in cultured cells.

5.5. Discussion

This chapter explores a nano-in-micro strategy to develop stimuli-responsive “turn on” theranostics. The microparticles displayed accelerated release in response to hyperthermia. Compared to the LCST microparticles we prepared in Chapter 4, UCST microparticles are more efficient in response to temperature because the internal drug diffusion kinetics increases in higher temperature. Hence, the drug release can be triggered by the UCST dissolution, as well as the quicker drug diffusion motivated by an increase of temperature. The nano-in-micro strategy further amplify microparticles’ sensitivity to heat, making it better thermo-responsiveness than the LCST microparticles.

Poly(Acrylamide-co-acrylonitrile) have been utilized to develop UCST drug delivery systems (e.g. micelle [4, 14], mesoporous silica particles grafting UCST ‘gate’ [12]). Most of them are nano-sale systems. Compared to their systems, our LDH-in-polymer microparticles are fabricated produced via a facile technique, spray drying. Since spray drying is a well-established, single-step process with benefits including good reproducibility and being easy to scale-up, the platform designed in this study could present a novel formulation strategy for theranostic applications. Additionally, the thermo-responsiveness $T_2$ relaxometry making them good temperature reporter during the hyperthermia.
5.6. Conclusions

In this work, we have successfully developed thermo-responsive theranostics for simultaneous MRI and controlled delivery of chemotherapeutic agents. These were generated by first incorporating drugs (5-fluorouracil and methotrexate, 5FU and MTX) into layered double hydroxide (LDH) nanoparticles, and then incorporating these with superparamagnetic iron oxide nanoparticles (SPIONs, MRI contrast agents) into in poly(acrylamide-co-acrylonitrile) microparticles. This polymer is thermoresponsive, and undergoes a globule-to-chain transition at around the physiological temperature. Microparticles with a concave shape were generated by spray drying, with elemental mapping indicating the SPIONs and LDH particles were homogenously distributed inside the formulations. The resultant nano-in-micro formulations displayed pH and thermosensitive in vitro drug release and relaxivity properties. Mathematical relationships could be elucidated to link drug release, temperature and the $r_2$ properties. In vitro cell culture models indicated that the microparticles can be used as thermo-responsive materials for hyperthermia-enhanced chemotherapy, with increased cell death noted when a short treatment at moderately elevated temperatures is paired with a chemotherapeutic. Hence, the formulations developed here could potentially be applied as theranostic platforms to improve therapeutic outcomes of hyperthermia and chemotherapy. This chapter has focused on $T_2$ MRI contrast agents, but advanced formulations are also required for $T_1$ imaging. This will be explored in Chapter 6.

5.7. References

Chapter 5

Thermo-responsive nano-in-micro particles


recurrent after mastectomy—a pilot experience. Oncology Research and Treatment, 27(4), 385-388.
Chapter 6

Gadolinium doped layered double hydroxides
6.1. Introduction

6.1.1. Layered Double Hydroxides in Biomedical Applications

Layered double hydroxide (LDH) nanocomposites have been widely explored in biomedical applications because of their biocompatibility, low toxicity and potential for cellular permeability. For example, hydrotalcite, a classic MgAl-LDH with the formula \([\text{Mg}_6\text{Al}_2(\text{CO}_3)(\text{OH})_{16} \cdot 4\text{H}_2\text{O}]\) has been commercialized by Bayer, with the product name of “Talcid®” [1]. It is prescribed for neutralizing the gastric acid of patients with acute or chronic gastritis.

A key point of LDH-based drug-delivery systems is that active agents intercalated can be released in a controlled manner at the target area. This capacity for localised sustained release can protect the agents from degradation and alleviate undesirable release outside the target site. This can minimise side effects, avoid toxicity and reduce dosing concentration and frequency, thereby improving the overall bioactivity [2, 3]. For instance, podophyllotoxin, a highly cytotoxic chemotherapeutic drug with poor water solubility and low bioavailability was intercalated into an LDH vehicle. Compared to the pure drug, *in vitro* cell experiments proved that the LDH nanocomposite enhanced the anti-cancer activity, suggesting that LDHs can promote cell penetration, increase the stability of the active agent, and release a drug payload locally in a controlled and sustained manner [4].

Another characteristic of LDH nanocomposite delivery systems is their pH sensitive properties. This is related to the nature of metal hydroxides. A number of experiments have revealed that in acidic conditions (e.g. pH 4.8, which mimics the environment in lysosomal conditions), the release of a drug from LDH-nanocomposites is faster than at the physiological pH (7.4) [2, 5]. According to Rives et al., drug release from the interlayer area is mainly dependent on two mechanisms: ion-exchange or weathering (dissolution of the layers) [6, 7]. At the physiological pH, it is driven by ion-exchange, while at acidic pH both ion-exchange and weathering are in play [8].

A wide range of active ingredients can be intercalated into the interlayer spaces of LDHs. For instance, DNA molecules contain negatively charged phosphate groups and can be intercalated into the lattice of an LDH by replacing interlayer nitrate ions via a ion-
exchange approach [9, 10]. These gene-LDH nanocomposites can thermodynamically stabilize the fragile DNA molecules, and prevent them from DNase degradation during the process of transportation into target cells. It was also found that vitamin C, which is unstable to light, oxygen, water or enzymes, can be stabilized in the LDH lattice [11, 12]. Compared to the free vitamin, LDH-vitamin C hybrids can prevent degradation in vivo and enable a higher percentage of the drug to infiltrate into epidermal tissues and reach deeper into the cells of the human skin [11, 12].

Methotrexate (MTX), a folate antagonist used in chemotherapy, suffers from drug resistance caused by reduced cell uptake, which is related to downregulation of uptake receptors in the tumour cell membrane. By intercalating MTX into the LDH interlayer, it can be transferred into the tumour cell via the uptake pathway of LDHs (typically a clathrin-mediated endocytic pathway), avoiding the issue of drug resistance [13, 14]. A wide range of other bioactive agents such as peptides and antibiotics can also be introduced into LDHs for advanced drug delivery applications [15].

### 6.1.2. Layered Double Hydroxides for MR Imaging

Nanomaterials are promising vehicles for use in theranostics because of their capacity to integrate different agents in a well-organized manner, and their potential to target a specific area of disease. LDHs can be easily doped with a number of other metal ions to obtain functional nanomaterials for magnetic resonance imaging (MRI) or computed tomography (CT). Further, the coordinating hydroxide groups can be protonated, providing a pH-responsive property which can be utilized in cancer diagnosis and treatment. A number of Gd-doped layered double hydroxides have been developed as multifunctional platforms for both bioimaging and treatment [16-18]. Other ions were also employed. For example, a manganese doped LDH was developed as a T1-MRI contrast agent sensitive to pH changes at the physiological range [19].

### 6.2. Aims and Objectives

In this chapter, Gd-doped LDHs (Gd-LDH) were fabricated as shown in Figure 6-1. The chemotherapeutic active pharmaceutical ingredients (APIs) MTX or 5-fluorouracil (5FU) were loaded into Gd-LDH via ion exchange. It was hypothesised that Gd-LDHs with appropriate size could be selectively delivered to tumour cells based on the
enhanced permeability and retention (EPR) effect, and once inside the cell the anticancer agents could be released and take effect. Meanwhile, the presence of Gd in the particles should mean that the local MRI contrast is also enhanced. Hence, these nanosheets might be used to deliver chemotherapeutics and simultaneously visualize the tumour and carrier location using T₁ relaxometry.

Figure 6-1 Schematic illustration of the production of drug loaded Gd-LDH particles.

The overarching objective of this chapter was to develop a smart nano-system for cancer theranostics, based on layered double hydroxides. The goals are as below:

- Synthesize and optimize Gd doped LDHs or ultra-thin LDHs, with the goal of obtaining a nanocarrier for MRI contrast and MTX or 5FU delivery.
- Explore the in vitro drug release and relaxation behaviour of the Gd-doped layered double hydroxides loaded with MTX or 5FU.

6.3. Experimental

6.3.1. Materials

Chemicals were sourced as follows: sodium hydroxide and anhydrous ethanol were purchased from Fisher Scientific Ltd. (UK); sodium chloride, xanthan gum, AlCl₃·6H₂O, MgCl₂·4H₂O, GdCl₃·6H₂O and fluorouracil (5FU) were obtained from Sigma-Aldrich.
(UK). Methotrexate (MTX) was purchased from Aladdin Co., Ltd. (China). Ultrapure water was collected from a Millipore MilliQ system operated at 18.2 MΩ.

6.3.2. Preparation of Layered Double Hydroxides (Mg₂Al-LDH)

The reaction was based on a previous report in the literature [20]. In a typical procedure, an aqueous solution (10 mL) containing MgCl₂·4H₂O (0.5 M, 167.3 g mol⁻¹, 0.8364 g) and AlCl₃·6H₂O (0.25 M, 241.4 g mol⁻¹, 0.6036 g) was quickly added into aqueous NaOH solution (40 mL, 0.4 M, 40 g mol⁻¹, 0.64 g) while stirring vigorously. After stirring for 20 min, the fresh Mg₂Al-LDH slurry was collected via centrifugation (9000 rpm, 20 min), washed twice with 20 mL of deionized water, and re-suspended in deionized water (40 mL). Then the white slurry was magnetically stirred (100 rpm) and heated at 100 °C in a 100 mL round-bottomed flask for different times, as detailed in Table 6-1.

<table>
<thead>
<tr>
<th>Table 6-1 The different heating times used to obtain Mg₂Al-LDHs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating time (h)</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

6.3.3. Preparation of Gd-doped Layered Double Hydroxides (Gd-LDHs)

A Mg₂Al-LDH slurry prepared with a Mg:Al molar ratio of 2:1 (as in Section 6.3.2) was resuspended in deionized water (30 mL). This suspension (A, 30 mL) was mixed with Solution B (GdCl₃·6H₂O, 371.7 g mol⁻¹, 7.5 mL, (details in Table 6-2) under stirring at room temperature for 12 h. The resultant slurry was separated by centrifugation, followed by washing with deionized water twice, and then re-suspended in deionized water (30 mL). After heat treatment at 100 °C (Table 6-2), the slurry was again centrifuged (9000 rpm, 20 min) and washed with deionized water three times. Finally, a homogeneous Gd-LDH suspension was obtained after re-dispersion in 10 mL of deionized water.
Table 6-2 The heating times and solution B compositions explored to obtain Gd-LDH

<table>
<thead>
<tr>
<th>Solution B (M)</th>
<th>B₁</th>
<th>B₂</th>
<th>B₃</th>
<th>B₄</th>
<th>B₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdCl₃·6H₂O (g)</td>
<td>0</td>
<td>0.005</td>
<td>0.01</td>
<td>0.025</td>
<td>0.05</td>
</tr>
<tr>
<td>Heating time (h)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6, 48</td>
<td>6</td>
</tr>
</tbody>
</table>

6.3.4. Bottom-up Synthesis of Gd-doped Layered Double Hydroxides (Gd-LDH-U)

In a typical procedure, 10 mL of an aqueous salt solution containing MgCl₂·4H₂O (0.5 M, 167.3 g·mol⁻¹, 0.8364 g), AlCl₃·6H₂O (0.237 M, 241.4 g·mol⁻¹, 0.5122 g), and GdCl₃ (0.013 M, 371.7 g·mol⁻¹, 0.0483 g) was quickly added into an aqueous NaOH solution (40 mL, 0.4 M, 40 g·mol⁻¹, 0.64 g) while stirring vigorously. After stirring for 20 min, 50 mL of the fresh Gd-LDH-U slurry was magnetically stirred (100 rpm) and hydrothermally treated at 110 °C for 4 h in a 100 mL round bottomed flask. Finally, the Gd-LDH-U slurry was centrifuged (9000 rpm, 20 min), washed with deionized water three times, and a transparent and homogeneous suspension obtained after re-dispersion in 10 mL of deionized water.

6.3.5. Preparation of ultra-thin layered double hydroxides (UT-LDH)

8 mL of an aqueous solution of Al(NO₃)₃·9H₂O (5 mM, 375.1 g·mol⁻¹, 0.0143 g) and Mg(NO₃)₂·6H₂O (10 mM, 256.4 g·mol⁻¹, 0.0205 g) and 6 mL of aqueous NaOH (15 mM, 40 g·mol⁻¹, 0.036 g) was added into 8 mL of a solution of NaNO₃ (5.2 mM, 85.0 g·mol⁻¹, 0.0035 g) in 25 % (v/v) aqueous formamide at 80 °C (magnetically stirred at 100 rpm). The resultant suspension was then heated for 30 min, washed by centrifugation using 20 mL of water: ethanol (1:1 v/v) three times (9000 rpm, 30 min), and then re-dispersed in 10 mL of deionized water.

Two different syntheses were explored here. In the first, 8 mL of a solution of Al(NO$_3$)$_3$·9H$_2$O (5 mM, 375.1 g mol$^{-1}$, 0.0143 g) and Mg(NO$_3$)$_2$·6H$_2$O (10 mM, 256.4 g mol$^{-1}$, 0.0205 g) in DI water and 6 mL of an aqueous solution of NaOH (15 mM, 40.0 g mol$^{-1}$, 0.036 g) were added into 8 mL of NaNO$_3$ (5.2 mM, 85.0 g mol$^{-1}$, 0.0035 g) in 25 % (v/v) aqueous formamide (stirred at 100 rpm) at 80 °C. This mixture was heated for 30 min in a 50 mL round bottomed flask. The resultant suspension was then washed by centrifugation using 20 mL of water: ethanol (1:1 v/v) three times. After centrifugation at 9000 rpm for 30 min, the pellet was taken and added to 6 mL of 50 % (v/v) aqueous formamide solution containing GdCl$_3$·6H$_2$O (1.5 mM, 371.7 g mol$^{-1}$, 0.004 g), and sonicating for 5 h. Ultra-thin Gd doped LDH was collected after washing the suspension three times using 20 mL of water: ethanol (1:1 v/v). This second synthesis yielded Gd-LDH-UT-1.

In the second synthesis, an 8 mL aqueous solution containing Al(NO$_3$)$_3$·9H$_2$O (4.25 mM, 375.1 g mol$^{-1}$, 0.0128 g), Mg(NO$_3$)$_2$·6H$_2$O (10 mM, 256.4 g mol$^{-1}$, 0.0205 g), and Gd(NO$_3$)$_3$·6H$_2$O (0.75 mM, 371.7 g mol$^{-1}$, 0.002 g) in DI water and 6 mL of aqueous NaOH (15 mM, 40.0 g mol$^{-1}$, 0.036 g) were added to 8 mL of NaNO$_3$ (5.2 mM, 85.0 g mol$^{-1}$, 0.0035 g) in 25 % (v/v) aqueous formamide at 80 °C (under stirring at 100 rpm). This mixture was heated for 30 min in a 50 mL round bottomed flask. The resultant suspension was then washed by centrifugation using 20 mL of water: ethanol (1:1 v/v) three times (9000 rpm, 30 min). Finally, the pellet was re-suspended in 10 mL of deionized water. This led to the formation of Gd-LDH-UT-2.
rpm). This mixture was heated for 30 min in a 50 mL round bottomed flask. The resultant suspension was then washed by centrifugation using 20 mL of water: ethanol (1:1 v/v) three times (9000 rpm, 30 min). Finally, the pellet was re-suspended in 10 mL of deionized water. This led to the formation of Gd-LDH-UT-2.

6.3.7. Characterization

Morphological Characterization

The morphology of the LDH nanosheets was characterised using SEM and TEM, using the protocols given in Section 2.4.1

Physicochemical Characterization

XRD, XPS and FTIR were performed as described in Section 2.4.2

Colloidal Properties

Hydrodynamic diameter and zeta potential measurements were conducted according to the protocols in Section 2.6.1


Gd-LDH-U from Section 6.3.4 was employed for these studies. 400 mg of Gd-LDH was suspended in 20 mL of water and 390 mg of 5FU or 455 mg of MTX was added to the solutions, keeping the pH at 9.5. After shaking at 40 °C for 48 h (100 rpm), 5FU or MTX-loaded Gd-LDHs (5FU-Gd-LDH or MTX-Gd-LDH) were collected after washing with water: ethanol (1:1 v/v) three times.

6.3.9. Stability Test

Composite stability was explored in PBS buffer (pH 6.5 or 7.4) and acetate buffer (pH 5.0). 50 mg of sample (n=3) was dispersed in 5 mL of the relevant buffer and transferred to a dialysis bag (MWCO=3500 Da). The bag was submerged into 15 mL of the same buffer and stirred at 37 °C (50 rpm). 1 mL aliquots were withdrawn from the medium at predetermined times and analyzed with an Agilent 7500cx inductively coupled plasma mass spectrometer (ICP-MS) to determine the free [Gd]. To maintain a constant
volume, 1 mL of fresh pre-heated buffer was added to the dissolution vessel at each time point.

6.3.10. In vitro Drug Release

Loading capacity (LC %) and encapsulation efficiency (EE %) were measured as detailed in Section 2.5.2.

Experiments were undertaken on a magnetic stirrer (C-MAG HS 7, IKA) with temperature control template. 20 mg samples (n=3) were dispersed in 5 mL of a buffer (pH 7.4 PBS buffer, or pH 5.0 acetate buffer) and transferred into a dialysis bag (MWCO=3500 Da). The bag was submerged into 15 mL of the same buffer and stirred at 37 °C (50 rpm). 0.3 mL aliquots were withdrawn from the dissolution medium at predetermined times and analyzed with an Agilent Cary 100 spectrophotometer. To maintain a constant volume, 0.3 mL of fresh pre-heated buffer was added to the dissolution vessel at each time point. MTX and 5FU quantifications were performed at λ_max of 303 nm for MTX and 262 nm for 5FU. Dilutions were undertaken when necessary to bring concentrations into the linear range of the calibration curve. Experiments were performed in triplicate and the results are reported as mean ± standard deviation (S.D.).

6.3.11. Proton Relaxivity Measurements

Proton relaxivity ($r_{1,2}$) were measured as described in Section 2.4.3.1. To monitor changes in proton relaxivity with pH, a dispersion of approximately 16 mg of each LDH sample in 8 mL of a 0.1 % (w/v) aqueous xanthan gum solution was placed into a 10 mm-diameter NMR tube, which was held at 37 °C. The longitudinal and transverse relaxation time ($T_1$ and $T_2$) were directly monitored over 7 h. At predetermined time points, 0.3 mL of suspension was taken from the NMR tube, diluted, and filtered through a PVDF-type syringe filter (0.22 μm). Then centrifugation (13,200 rpm for 10 min) was conducted to remove the remaining particles, and the drug concentration and free [Gd] were determined using Uv-vis and ICP-MS, respectively. All experiments were performed in triplicate and the results are reported as mean ± S.D.
6.4. Results

6.4.1. Gd Doped LDHs

6.4.1.1. Mg₂Al-LDH

Mg₂Al-LDH was synthesized via co-precipitation with different aging times (6, 10, 24 h; Mg₂Al-LDH-1, 2 and 3 respectively).

XRD

The peaks in the XRD patterns (Figure 6-2) can be indexed to the LDH (003), (006), (009), (015), (018), (110), and (113) reflections, revealing the presence of a well crystalized hydrotalcite (JCPDS card 35-0965). The sharper peaks observed with longer aging times indicate that the degree of crystallinity was enhanced.

![Figure 6-2 XRD patterns of Mg₂Al-LDH aged for 6, 10, 24 h.](image)

Hydrodynamic Diameters

The hydrodynamic diameters of the three Mg₂Al-LDH samples (Figure 6-3 and Table 6-3) were measured by dynamic light scattering (DLS). The size of Mg₂Al-LDH particles clearly increases when prolonging the heating time.
Figure 6-3 Size distributions of Mg2Al-LDH particles aged for 6, 10, 24 h. Representative datasets from three independent measurements are shown.

These results showed that the size and degree of crystallinity of the LDH particles are highly related to the reaction time. Hence, we will explore a series of reaction times as well as [Gd] in the preparation of Gd doped LDHs.

Table 6-3 Hydrodynamic diameters of Mg2Al-LDH prepared at different aging times. Data represented as mean ± S.D. from three measurements.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Z-average (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg2Al-LDH-1</td>
<td>85 ± 2</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Mg2Al-LDH-2</td>
<td>195 ± 5</td>
<td>0.18 ± 0.00</td>
</tr>
<tr>
<td>Mg2Al-LDH-3</td>
<td>694 ± 41</td>
<td>0.34 ± 0.01</td>
</tr>
</tbody>
</table>

6.4.1.2. Gd Doped LDH

Gd-LDH (Table 6-2) was first synthesized using a two-step method (Scheme 6-1A): an initial co-precipitation approach to prepare Mg2Al-LDH and then subsequent partial isomorphic substitution of Al3+ with Gd3+. Gd-LDHs with different aging times were produced and denoted as Gd-LDH-Bn. A second ‘one-step’ method was also explored (Scheme 6-1B). In this, Gd-LDH was synthesized via a small amount of Gd3+ being added into the Mg/Al solution before co-precipitation. The Gd-LDH synthesized via this bottom-up approach was denoted Gd-LDH-U.
Scheme 6-1 Preparation of Gd-doped LDH via (A) two-step or (B) bottom-up approaches.

**XRD**

**Figure 6-4** displays the powder XRD pattern of Gd-doped LDHs prepared via the two-step method (Gd-LDH-Bn) with 6 h aging. The layered structure of the LDH is retained after gadolinium doping, and characteristic Bragg reflections of an LDH phase are present in the XRD patterns (**Figure 6-4** cf. **Figure 6-2**). However, new peaks were found as the Gd ratio increased, which can be indexed to the (100), (110), (101), (020), (201), (120), (300), (002) and (220) reflections of hexagonal Gd(OH)₃ (JCPDS card 38-1042). The XRD patterns indicate that gadolinium doping via a two stage process results in Gd(OH)₃ impurities being formed at higher Gd concentrations.

In contrast, for Gd-LDH-U only the characteristic reflections of the LDH phase can be observed, and no obvious reflection of Gd(OH)₃ are present. However, the crystallinity of Gd-LDH-U is lower than with the other systems.
Chapter 6

Gadolinium doped layered double hydroxides

Figure 6-4 XRD patterns of Gd-doped LDHs with varied Gd contents (Gd/Mg molar ratio for B<sub>1-5</sub>: 0, 1 : 133, 1 : 67, 1 : 27, 1 : 13.3, respectively; 1 : 38 for Gd-LDH-U; heating time 6h) and Gd(OH)<sub>3</sub> reference (JCPDS card 38-1042).

Further investigation was undertaken to explore the effect of aging time on Gd-LDH-B<sub>4</sub>. According to the XRD data (Figure 6-5), the well-defined basal reflections of the LDH phase were decreased and partly replaced by broad peaks when increased aging time was increased to 48 h. This indicates that the hydrotalcite-like structure was disrupted and disorder introduced into layer stacking. At the same time, the reflections of Gd(OH)<sub>3</sub> become more intense, indicating that this phase becomes dominant at longer aging times. Thus, the optimal heating time can be taken to be 6 hours.
Colloidal Properties

Size and Zeta Potential

The blank LDH displayed a mean size of $75 \pm 2$ nm, and an increasing Gd content results in larger particle sizes (Figure 6-6 and Table 6-4). The mean size of Gd-LDH-U (Gd/Mg molar ratio of 1 : 38) was $186 \pm 1$ nm with PDI of $0.24 \pm 0.00$. Compared to Gd-LDH-B$_4$ with a Gd/Mg molar ratio of 1 : 27, Gd doped LDHs synthesized via the bottom-up approach possess larger particle sizes. All the particles should be suitable for the intended application, since it is generally believed that small particles (<200–300 nm) can be selectively accumulated in a tumour based on the EPR effect [21-23].
Similarly, the zeta potential of the Gd-LDHs increased with the content of Gd$^{3+}$ ions (Table 6-4). This is to be expected, given the increased charge density that will result from greater incorporation of the higher charged Gd ions in the LDH layers. All the zeta potential values noted are likely to result in stable colloids, with the particles like to remain isolated and available for cellular uptake (rather than flocculating). To validate this hypothesis, the colloidal stability was explored, and photographs are presented in Figure 6-7. Gd-LDH-B$_4$ and Gd-LDH-U remained stable over at least 5 days, similar to Mg$_2$Al-LDH. Gd-LDH-B$_4$ and Gd-LDH-U were thus selected for further investigation since they possess desirable size with excellent colloidal stability, and their Gd/Mg molar ratios are similar.

**Table 6-4** Hydrodynamic diameters, PDI, and zeta potentials of Gd-doped LDHs (~ 1 mg/mL). Data presented as mean ± S.D. from three measurements.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Z-average (nm)</th>
<th>PDI</th>
<th>Z-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-LDH-B$_1$</td>
<td>75 ± 2</td>
<td>0.19 ± 0.01</td>
<td>+40 ± 1</td>
</tr>
<tr>
<td>Gd-LDH-B$_2$</td>
<td>91 ± 1</td>
<td>0.21 ± 0.01</td>
<td>+46 ± 0</td>
</tr>
<tr>
<td>Gd-LDH-B$_3$</td>
<td>98.2 ± 1</td>
<td>0.33 ± 0.01</td>
<td>+53 ± 1</td>
</tr>
<tr>
<td>Gd-LDH-B$_4$ (6h)</td>
<td>124 ± 1</td>
<td>0.25 ± 0.01</td>
<td>+54 ± 1</td>
</tr>
<tr>
<td>Gd-LDH-B$_5$</td>
<td>220 ± 3</td>
<td>0.37 ± 0.01</td>
<td>+59 ± 1</td>
</tr>
<tr>
<td>Gd-LDH-U</td>
<td>186 ± 1</td>
<td>0.29 ± 0.00</td>
<td>+49 ± 1</td>
</tr>
</tbody>
</table>
Delamination of an LDH particle can be used as a route for producing positively charged platelets with a thickness of a few atomic layers. These can be used as nanocomposite additives for polymers, or as building units for making new organic–inorganic or inorganic–inorganic nanomaterials [24]. The small particle size and narrow thickness of the particles might enable more water access to paramagnetic centres, which could in turn enhance the contrast ability. In this section, two different ultra-thin Gd-doped LDH samples were prepared. In the first method, ultra-thin LDH particles were prepared via formamide exfoliation and then doped with gadolinium (UT-Gd-LDH-1, Scheme 6-2A). The second approach is to firstly generate Gd-LDH via a bottom-up method, and subsequently exfoliate using formamide (UT-Gd-LDH-2, Scheme 6-2B).

Scheme 6-2 Preparation of ultra-thin Gd-doped LDH via (A) two-step ((B) single-step approaches.

Figure 6-7 Photographs of Gd-LDH-B4 (6h, left) and Gd-LDH-U (right) suspensions (~ 1 mg/mL) over a 5 day storage period.

6.4.1.3. Ultra-thin Layered Double Hydroxides (UT-LDH)
XRD

Characteristic Bragg reflections of LDHs can be identified in the XRD patterns of UT-LDH and UT-Gd-LDH-1 (Figure 6-8). The broad peaks of UT-Gd-LDH-2 suggest a nanocrystalline material with low crystallinity. No reflections of Gd(OH)$_3$ impurities are observed. This could be because the phase is absent, but might also be a result of the low degree of crystallinity caused by use of a shorter heating time in this method.

![XRD pattern of ultra-thin Gd-doped LDHs](image)

Figure 6-8 XRD patterns of ultra-thin Gd-doped LDHs.

Colloidal Properties

Size and Zeta Potential

The particle size of the UT-LDHs was investigated. As shown in Table 6-5, the addition of Gd ions results in an increase in particle size and zeta potential. These results are consistent with those obtained previously in this chapter.

Table 6-5 Hydrodynamic diameter and zeta-potential of ultra-thin Gd-doped LDH (~ 1 mg/mL). Data represented as mean ± S.D. from three measurements.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Z-average (nm)</th>
<th>PDI</th>
<th>Z-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-LDH</td>
<td>95 ± 1</td>
<td>0.22 ± 0.02</td>
<td>+39 ± 2</td>
</tr>
<tr>
<td>UT-Gd-LDH-1</td>
<td>130 ± 2</td>
<td>0.21 ± 0.01</td>
<td>+43 ± 1</td>
</tr>
<tr>
<td>UT-Gd-LDH-2</td>
<td>168 ± 1</td>
<td>0.23 ± 0.01</td>
<td>+44 ± 1</td>
</tr>
</tbody>
</table>
Colloidal Stability

The colloidal stability of the UT-LDHs is seen to be high, as is evidenced by the images in Figure 6-9.

\[ \text{UT-Gd-LDH-1} \quad \text{UT-Gd-LDH-2} \]

Figure 6-9 Photographs of UT-Gd-LDH-1 (left) and UT-Gd-LDH-2 (right) suspensions (~ 1 mg/mL) over 5 days.

6.4.1.4. X-ray Photoelectron Spectroscopy

To further explore the presence of gadolinium in the formulations, X-ray photoelectron spectroscopy (XPS) was carried out. The detailed Gd 4p spectra are given in Figure 6-10.

Figure 6-10 High resolution Gd 4p XPS spectra of (A) Gd-LDH-Ba, (B) Gd-LDH-U, (C) UT-Gd-LDH-1 and (D) UT-Gd-LDH-2.
The spectra of Gd-LDH-B$_4$ and Gd-LDH-U revealed strong peaks around 143 eV and small peaks at 149 eV, which can be assigned to Gd 4d$_{5/2}$ and 4d$_{3/2}$, respectively [25]. For UT-Gd-LDH-1 and UT-Gd-LDH-2, strong peaks related to 4d$_{5/2}$ (143 eV) and 4d$_{3/2}$ (149 eV) also can be observed. The peaks of Gd 4d$_{3/2}$ (149 eV) of UT-Gd-LDH-1 and 2 are more intense compared to those of Gd-LDH-B$_4$ and Gd-LDH-U, indicating more Gd$^{3+}$ represented in ultra-thin systems.

6.4.1.1. Morphological Characterization

The morphology of the Gd doped LDHs and drug loaded Gd-LDH-U was studied using TEM. The results revealed that Gd-LDH-B$_4$, Gd-LDH-U, UT-Gd-LDH-1 and UT-Gd-LDH-2 consist of pseudo-hexagonal nanosheets (Figure 6-11), a typical morphology for LDH particles [26]. Mean particle sizes are 63 ± 24, 81 ± 25, 58 ± 19 and 76 ± 25 nm, respectively. These values size are lower than the hydrodynamic diameter from DLS. This is expected because the hydrodynamic diameter values include the solvation layers [27, 28].

Compared to other three Gd doped LDHs, small nanoparticles (less than 5 nm) can be observed on the surface of Gd-LDH-B$_4$ (Figure 6-11B). These could potentially correspond to the Gd(OH)$_3$ impurities found in the XRD patterns.
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Figure 6-11 TEM images and size distribution of (A-C) Gd-LDH-B$_4$ with aging time of 6 h, (D-F) Gd-LDH-U, (G-I) UT-Gd-LDH-1 and (J-L) UT-Gd-LDH-2.

Gd-LDH-B$_4$ with aging time of 48 h was also examined with TEM (Figure 6-12). Small dots can be found on the surface of thick particles, while the typical pseudo-hexagonal structure of LDHs was difficult to observe. The TEM results are as consistent with previous XRD results (see Figure 6-5).
Figure 6-12 TEM images of Gd-LDH-B4 with aging time of 48 h (scale bar: 500 nm).

6.4.2. Relaxivity Measurements

To find the system best able to act as a contrast agent, the relaxivity ($r_1$) of selected samples was measured. The $r_1$ values of a clinically used T1-MRI contrast agent was also measured for comparison. The results are given in Table 6-6. All particles possess good T1 contrast agent properties, with a $r_2/r_1$ ratio between 1.15 to 1.23. Compared to the clinical standard Gd(DTPA), the $r_1$ relaxivity value is notably higher with the LDH particles, indicating their potential as contrast agents for clinical applications.
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Table 6-6 Relaxivity of different Gd-LDHs and UT-Gd-LDHs (n=3)

| Sample          |  
|-----------------|-----------------|-----------------|
|                 | $r_1$ (mM$^{-1}$s$^{-1}$) | $r_2$ (mM$^{-1}$s$^{-1}$) | $r_2/r_1$ |
| Gd(DTPA)        | 4.5 ± 0.6        | 5.0 ± 0.8        | 1.2        |
| Gd-LDH-B$_4$    | 12.8 ± 1.6       | 15.7 ± 2.1       | 1.23       |
| Gd-LDH-U        | 9.5 ± 1.2        | 11.0 ± 1.8       | 1.16       |
| UT-Gd-LDH-1     | 14.0 ± 2.1       | 16.4 ± 2.3       | 1.18       |
| UT-Gd-LDH-2     | 13.2 ± 1.7       | 15.8 ± 1.4       | 1.15       |

UT-Gd-LDHs possess higher relaxivity compared to Gd-LDHs. This might be because they have thinner particles which allow more water protons to coordinate to the paramagnetic Gd$^{3+}$ centre. The relaxivity of Gd-LDH-U is relatively low since some of the Gd$^{3+}$ is located inside the nanoparticles as a result of the bottom-up synthesis used. For Gd-LDH-B$_4$, the Gd$^{3+}$ doping and Gd(OH)$_3$ impurity are expected to be distributed on the surface, leading to higher relaxivity. The ultra-thin LDHs possess slightly greater relaxivity, but their synthesis is complicated and requires a large amount of solvent, which makes further experiments challenging. Even though Gd-LDH-B$_4$ possess higher relaxivity, it contains Gd(OH)$_3$ impurities and two steps were required for preparation, leading to a loss of final products. In contrast, Gd-LDH-U can be obtained in one-step and also displayed good T$_1$ relaxivity, higher than the clinical used MRI contrast agent Gd(DTPA). From the perspective of maximizing the synthetic efficiency, Gd-LDH-U was thus selected for onward studies.

6.4.3. Drug Loading Gd-LDHs

To obtain theranostics for MRI-guided chemotherapy, two chemotherapeutics, 5FU and MTX were loaded into pre-formed Gd-LDH-U via ion-exchange.

6.4.3.1. XRD

Compared to the XRD patterns of blank Gd-LDH-U, the (003) and (006) reflections (Figure 6-13) are broader and lower intensity after MTX or 5FU intercalation, as a result of the introduction of stacking defects [29, 30]. The (003) peak is also shifted towards lower angle because of an increase in the interlayer spacing to accommodate the larger drug ions [29, 30]. No reflections of the APIs (Figure 5-8) are observed in the XRD
data, suggesting drug were intercalated into the metal layers and no pure drug crystals are present.

![XRD patterns](image)

Figure 6-13 XRD patterns of Gd-LDH-U, and Gd-LDH-U loaded with 5FU or MTX.

### 6.4.3.2. TEM

After drug loading, the LDHs manifest as aggregated plate-like nanoparticles (Figure 6-14). This is consistent with the literature [31].

![TEM images](image)

Figure 6-14 TEM images of (A) 5FU-Gd-LDH and (B) MTX-Gd-LDH.
6.4.3.3. **DSC**

No endothermic melting peaks can be found in DSC profiles (Figure 6-12), confirming that there are no MTX or 5FU crystals present in the reaction product.

![DSC profile](image)

Figure 6-15 DSC profile of the drug loaded Gd-LDH samples and raw materials.

6.4.3.4. **Hydrodynamic Diameter**

The hydrodynamic diameter of 5FU-Gd-LDH slightly increased to 303 ± 4 nm (PDI 0.28 ± 0.01), while MTX-Gd-LDH displayed a larger particle size (416 ± 4 nm, PDI 0.64 ± 0.01).

6.4.3.5. **IR Spectra**

FTIR spectra are shown in Figure 6-13. The stretching bands of both hydroxide and interlayer water molecules are found at approximately 3400 cm\(^{-1}\), and a band at 1647-48 cm\(^{-1}\) can be ascribed to the δ-bend of water. The low intensity peak at around 1450 cm\(^{-1}\) in MTX-Gd-LDH can be attributed to stretching vibration of C=C bonds in MTX, and the peak at 1610 cm\(^{-1}\) corresponds to the water bending mode. Further, a pair of absorption bands at 1387 and 1558 cm\(^{-1}\) arise from COO\(^{-}\) stretching vibrations of the carboxylic acid groups of MTX. Similarly, successful intercalation of 5FU was
confirmed. There are a range of additional peaks characteristic of 5FU in 5FU-Gd-LDH. These arise around 1672 cm$^{-1}$ (C=O and C=C stretches), 1586–1300 and 809 cm$^{-1}$ (ring stretching modes) [45], and 1216 cm$^{-1}$ (C–F vibrations) [32].

![Figure 6-16 FTIR spectra of MTX-Gd-LDH, 5FU-Gd-LDH, pristine Gd-LDH, MTX and 5FU.](image)

**6.4.4. Stability Test**

Free Gd$^{3+}$ is toxic to humans [33]. Thus the stability of the nanocomposites was explored at pH 5.0 (late endosome/lysosome in cells), pH 6.5 (tumour microenvironment) and pH 7.4 (representing the general physiological environment). Experiments were performed for 24 h, and the [Gd] freed into the release medium was analyzed using ICP-MS. The results are given in Figure 6-17.
Figure 6-17 [Gd] released from (A) MTX-Gd-LDH and (B) 5FU-Gd-LDH at pH 6.5 and 5.0. Data are given from three independent experiments as mean ± S.D.

At pH 7.4, no Gd was detected, indicating the drug loaded Gd-LDHs are stable in neutral environments. At pH 6.5 and 5.0, a small proportion of [Gd] was released after 24 h, equating to a maximum of 2.4 (pH 6.5) and 2.9 % (pH 5.0) of the Gd content leaching for MTX-Gd-LDH, and 1.9 (pH 6.5) and 2.4 % (pH 5.0) for 5FU-Gd-LDH, respectively. Hence, the Gd-LDH nanosheets are stable in most physiological environments (pH > 7.0), but free Gd$^{3+}$ ions might be freed in acidic environments (e.g. tumour environments, GI tract) in vivo. This could be a risk for patients.

### 6.4.5. **In vitro Dissolution Studies**

#### 6.4.5.1. **LC and EE**

The drug loadings of MTX-Gd-LDH and 5FU-Gd-LDH were calculated to be $34.6 \pm 5.9$ and $13.2 \pm 6.2$ w/w % (mean ± SD, n=5), with encapsulation efficiency (EE) of $30.4 \pm 5.2$ and $13.5 \pm 6.4$ %, respectively.

#### 6.4.5.2. **In vitro Release Profiles**

Drug release was explored at pH 5.0, 6.5 and 7.4. The results are presented in Figure 6-18. A burst release of drug from the LDH can be found in all cases.

The LDHs exhibit pH-responsive release rates. For MTX-Gd-LDH, the final release percentage (48 h) is around 80 % at pH 5.0, 67 % at pH 6.5, and 57 % at pH 7.4. The release of 5FU-Gd-LDH reached the highest release percentage after around 7 h (approx. 88 % at pH 75 % at pH 6.5 and 70 % at pH 7.4), following by a slight decline to ca.
86% (pH 5.0), 73% (pH 6.5) and 67% (pH 7.4) after 48 h, likely to be due to some drug adsorbing back onto the LDH composite [29].

![Diagram showing drug release profiles of (A) 5FU-Gd-LDH and (B) MTX-Gd-LDH.](image)

**Figure 6-18** Drug release profiles of (A) 5FU-Gd-LDH and (B) MTX-Gd-LDH. Data are given from three independent experiments as mean ± S.D.

### 6.4.5.3. Release Kinetics

The Bhaskar ([Equation 2-4](#)) [34] and Avrami-Erofe'ev models ([Equation 2-5](#)) [35] were used to fit the release data. Detailed information on these models can be found in Section 2.6.1.

**Bhaskar Model**

Fits of the Bhaskar model to the release data are shown in **Figure 6-19**. The release data in general can be well fitted to this model in all three pH environments. The Bhaskar equation assumes that the that drug release rate is dependent on the diffusion of the drug ions and/or replacement anions through the system. Thus most drug release (~70%) can be regarded to be an ion-exchange controlled process, which occurred in the first two hours.
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Figure 6-19  Fits of the Bhaskar equation to the first 2 h (no more than 70 %) of release data for 5FU-Gd-LDH at (A) pH 7.4, (B) 6.5, (C) 5.0, and MTX-Gd-LDH at (D) pH 7.4, (E) 6.5, and (F) 5.0.

Avrami-Erofe'ev Model

As shown in Figure 6-20 and Table 6-7, the Avrami-Erofe'ev model also fits the release data. The value of $n$ provides some information on the reaction mechanism. The values of $n$ lie in the range of 0.37 to 0.60. All of these suggest diffusion control, indicating that diffusion of replacement ions through the solution/interlayer space limits the rate. This is consistent with the fitting results obtained with the Bhaskar model.
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Figure 6-20 Fits of the Avarmi-Erofe’ev model to the data for 5FU-Gd-LDH at pH (A) 7.4, (B) 6.5, (C) 5.0, and MTX-Gd-LDH at (D) pH 7.4, (E) 6.5, and (F) 5.0.

Table 6-7 The values of the $n$ exponent and $R^2$ determined from the Avarmi-Erofe’ev model, using the data obtained from the first 2h (~60 %) of drug release.

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>$n$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FU-Gd-LDH</td>
<td>7.4</td>
<td>0.58</td>
<td>0.9810</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>0.58</td>
<td>0.9841</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.56</td>
<td>0.9952</td>
</tr>
<tr>
<td>MTX-Gd-LDH</td>
<td>7.4</td>
<td>0.60</td>
<td>0.9700</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>0.37</td>
<td>0.9090</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.50</td>
<td>0.9837</td>
</tr>
</tbody>
</table>

Summary

Of the model considered, the Peppas and Bhaskar model gave reasonable $r^2$ values – all above 0.90. This indicates that the release of the intercalated therapeutic agents (5FU and MTX) from the Gd-LDH lattice is a type of heterogeneous diffusion processes, which would be associated with the drug release from the LDH layer producing many heterogeneous sites. The $n$ values determined from Avrami analysis are ca. 0.5, which is consistent with diffusion control. However, it should be noted that there are a range of potential interpretations of the $n$ values obtained in Avrami model, and thus it is not possible to draw definitive conclusions here.
6.4.6. Relaxivity Monitoring

To explore the MRI properties of the drug loaded Gd-LDHs, we investigated the relaxation behaviour of 5FU-Gd-LDH and MTX-Gd-LDH at different pHs over 24h. The free [Gd] was also monitored to elucidate any relationship between [Gd] and proton relaxivity.

6.4.6.1. \( r_1 \) Profiles

As shown in Figure 6-21A, the \( r_1 \) values of MTX-Gd-LDH remained low and roughly unchanged at neutral pH (~ 0.83 mM s\(^{-1}\)), while in acidic environments the \( r_1 \) values are higher, increasing to 2.3 mM s\(^{-1}\) at pH 6.5 and 2.7 mM s\(^{-1}\) at pH 5.0 after 24 h. The \( r_1 \) profiles of 5FU-Gd-LDH showed similar trends (Figure 6-21B).
Figure 6-21 The longitudinal relaxivity ($r_1$) profile of (A) 5FU-Gd-LDH and (B) MTX-Gd-LDH, with drug release profiles of (C) 5FU-Gd-LDH and (D) MTX-Gd-LDH, and [Gd] release from (E) 5FU-Gd-LDH and (F) MTX-Gd-LDH.

6.4.6.2. Drug and [Gd] Release

The corresponding drug and [Gd] freed into medium are given in Figure 6-21C-F. Both drug loaded Gd-LDHs displayed higher relaxivity ($r_1$) in acidic pH since a small proportion of Gd was released into the acidic buffers, thus promoting interactions between Gd$^{3+}$ paramagnetic centres and water protons. The pH-sensitive relaxation
behaviour potentially could allow the particles to be used for reporting on the acidic tumour microenvironment in the human body.

6.5. Discussion

LDH materials are protective and controlled delivery vehicles that have attracted much attention in pharmaceutical science. These materials can be easily doped with a number of other metal ions to obtain functional nanomaterials for MRI or computed tomography.

In this study, four different Gd-doped LDH nanosheets were synthesized using different methods. All displayed reasonable relaxivity. XRD patterns of Gd-LDH nanosheets prepared via a two-step process clearly revealed the existence of Gd(OH)$_3$ impurities. This is further supported by TEM images, as both pseudo-hexagonal LDH nanosheets and smaller nanoparticles with distinct morphologies are presented. These findings may be a result of the size of the Gd$^{3+}$ cation (118 pm), which is much larger than Al$^{3+}$ ion (68 pm) [36]. As a result, Gd$^{3+}$ cannot be accommodated in the octahedral holes occupied by Al$^{3+}$, preventing or minimising isomorphic substitution. Hence, the surfaces of substituted LDHs were likely to have adsorbed Gd and, after thermal treatment, crystallized Gd(OH)$_3$ particles formed on the surface of LDHs. While for Gd-LDH-U, prepared in a single step, the Gd ions were evenly distributed inside the LDH particles, preventing the formation of separate Gd(OH)$_3$ particles. For the ultra-thin Gd-LDHs, no Gd(OH)$_3$ impurities were found in XRD and TEM. This might be because of complete incorporation in the LDH layers, and/or arise because the Gd hydroxide phase is present but amorphous as a result of using a shorter thermal treatment with lower temperature.

The drug loaded Gd-LDH-U nanosheets displayed rapid release profiles, reaching a plateau after ~3 hours, consistent with previous reports in the literature [29, 37]. The release is also pH-responsive, and more drug was freed in acidic conditions as a result of the LDH layers weathering in the presence of H$^+$ [38]. Compared to pristine Gd-LDH-U, the longitudinal relaxivity ($r_1$) values decreased after drug loading. This might be because the drug ions intercalated are more hydrophobic than the original guest anions (Cl$^-$), weakening the corporation between paramagnetic centre (Gd$^{3+}$) and water protons. However, the $r_1$ values (~1-4 mM·s$^{-1}$) are still relatively close to Gd(DTPA) (4.5 mM·s$^{-1}$), indicating acceptable $T_1$ MRI contrast ability. There is only minimal change in $r_1$
with time, which means that it is not possible to use these formulations to directly track drug release, as has been possible in previous chapters.

### 6.6. Conclusions

Four different MgAl LDH formulations doped with Gd$^{3+}$ were synthesized and fully characterised by X-ray diffraction, IR spectroscopy and relaxivity measurements. The successful doping of Gd$^{3+}$ was evidenced by XPS spectra showing characteristic peaks of Gd (4p). All these systems display good $r_1$ relaxivity and thus can potentially be used to develop MRI-based theranostics. A Gd$^{3+}$ doped LDH prepared by a ‘bottom-up’ method was selected to load with chemotherapeutic APIs (5FU and MTX), since it can be obtained in one step and had acceptable relaxivity. 5FU and MTX were loaded via ion-exchange. X-ray diffraction patterns exhibit expanded interlayer spacings as a result of successful intercalation of APIs. IR spectra also showed characteristic peaks of MTX or 5FU. The stability of Gd doped in LDHs was found to be high under physiological conditions, while in acidic conditions a small proportion of Gd was freed into the immersion medium. Dissolution tests revealed that both 5FU and MTX rapidly released from the LDH carrier, in a manner slightly responsive to acidic pH. The longitudinal relaxivity of Gd-LDHs remains roughly stable during drug release over 24h, and was higher in acidic environments. Overall, the drug-loaded Gd-LDH systems prepared in this chapter could serve as pH-sensitive theranostic platforms for MRI-guided anticancer therapy.

### 6.7. References


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materials with magnetic resonance response as magnetic resonance/fluorescence bimodal probes. Optical Materials, 57, 56-62.


Chapter 7

Polydopamine-coated theranostic implants
7.1. Introduction

7.1.1. Implantable Drug Delivery Systems

Postoperative adjuvant chemotherapy has been proven to improve long-term prognosis in the treatment of early and localized tumours compared to surgery alone [1, 2]. However, systematic chemotherapy often fails to achieve desirable clinical outcomes due to the presence of numerous drug delivery barriers in the body. For example, patients with pancreatic ductal adenocarcinoma (PDAC) often suffer from chemotherapy resistant tumours due to a barrier of fibrous tissue preventing drug transport into the tumour site [3, 4]. The blood brain barrier hampers drug accumulation in the target area in glioma patients, increasing the risk of tumour recurrence and metastasis [5]. In these circumstances, alternative delivery approaches are required to enhance chemotherapy efficacy.

A novel therapeutic option to achieve this is to use implantable drug delivery (IDD) systems. Such formulations address issues associated with oral and intravenous administration by directly delivering chemotherapy into a local tumour site over a prolonged period of time (Figure 7-1) [6]. The advantages of IDD systems are as follows:

- Local drug delivery, reducing the required drug dosage and the side/off-target effects.
- Prolonged release profiles.
- Protecting the loaded drugs from degradation or clearance until they are released.
- Overcoming physiological and pathological barriers, and conquering associated drug resistance.
Figure 7-1 Schematic of systemic drug delivery versus local drug delivery for the treatment of malignant tumours, illustrating various types of implantable polymeric systems and different anticancer therapeutic modalities which can be employed. Reproduced with permission [6]. Copyright © 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Indolfi et al. reported an implantable poly(lactic-co-glycolic)-based biodegradable device to achieve local paclitaxel therapy for PDAC patients. Compared to systemic therapy, their device could increase the efficacy of suppression of local tumour growth using an orthotopic xenograft model in mice [7]. Xie fabricated an implantable curcumin-based nanofibrous matrix for treatment of a solid tumour. After an ethanol vapour treatment, the formulation displayed prolonged drug release and improved intracellular drug uptake [8]. There is further a clinically successful implantable wafer,
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Gliadel™, which is placed into the tumour resection cavity and provides a specific post-surgical solution for glioma patients.

Electrospun fibres have attracted increasing attention as implantable theranostic platforms [9, 10]. For example, Ramachandran fabricated an electrospun implant which could continuously release temozolomide for up to one month, and simultaneously allowed monitoring of potential side effects caused by implantation for up to 3 months [11]. In most such studies to date, functional agents are encapsulated inside the fibres. Little research has been reported using post-fabrication modification to aid diagnosis and therapy.

7.1.2. Polydopamine Modification

A powerful strategy to enhance the features of implants without compromising the inherent properties of the component materials is surface functionalization. Polydopamine (PDA) coating has recently been widely explored as a facile, one-step approach for this purpose. Coating can be achieved by the polymerisation of dopamine monomers. The mechanism behind this polymerisation is complex (Figure 7-2) and is to date not fully understood [12-14].

![Figure 7-2 Polymerization and interaction mechanisms of dopamine. A) Polydopamine formation: the copolymer consisting of 5,6-dihydroxyindole (DHI) and dopamine. Reproduced with permission [12]. © 2020 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.](image)

PDA is a biocompatible and biodegradable biomaterial which has attracted extensive attention in the area of biomedicine [15]. With a melanin-mimicking structure and rich
phenol groups, PDA materials display excellent free radical scavenging abilities, and can serve as antioxidant and anti-inflammatory agents for injury healing and suppression of implant-related immune responses [16, 17]. The large amount of phenol groups provide good chelating capabilities and can be employed to bind metal ions such as radioisotope ions for nuclear imaging [18, 19]. The ability of PDA to absorb versatile molecules has also been explored and potential applications identified in the areas of drug delivery, molecular imaging and cancer theranostics [18-20]. Moreover, it is reported PDA materials have moderate antibacterial effects, desirable for implant systems to reduce the attachment of and kill bacteria [21].

7.2. Aims and Objectives

In this chapter, implantable fibres incorporating MTX and MRI contrast agents were developed, as shown in Scheme 7-1. To enhance MTX stability and prolong the drug release profile, MTX was firstly loaded into a LDH nanocarrier. IDD systems comprising biodegradable polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA) based core-shell fibres loaded with LDH-MTX were then produced by electrospinning (Figure 7-3). MRI contrast agents (SPIONs or Gd(DTPA)) were subsequently incorporated into the formulation via post-fabrication polydopamine coating.

The overarching objective of this chapter was to develop PDA-coated nanocomposite theranostic implants for MRI-guided localized chemotherapy. The goals are as below:

- Fabricate PCL@PLGA or PCL@PCL fibres loaded with MTX loaded LDH nanoparticles.
- Incorporate MRI contrast agents (SPIONs or Gd(DTPA)) onto the fibres via post-fabrication PDA coating.
- Characterize the coated nanocomposite fibres.
Scheme 7-1 Schematic illustration of the fabrication of implantable fibre-based theranostics.

Figure 7-3 The chemical structures of PCL (A) and PLGA (B).
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7.3. Experimental

7.3.1. Materials

Two different forms of poly(D,L-lactide-co-glycolic)acid (PLGA) were purchased: PLGA-1 (acid terminated, 150 kDa, lactide:glycolide 75:25) and PLGA-2 (ester terminated, 80 kDa, lactide:glycolide 50:50) were obtained from Jinan Daigang Biomaterial Co., Ltd. (China). Polycaprolactone (PCL; 80 kDa), Gd(DTPA)·2H₂O, crystal violet were obtained from Sigma Aldrich (UK), Dichloromethane, N,N-dimethylformamide (DMF), MgCl₂·6H₂O, AlCl₃·6H₂O, and sodium hydroxide were purchased from Macklin Co., Ltd. (China). MTX and Tris buffer were sourced from Aladdin Co., Ltd. (China). Ultrapure water (> 18.2 MΩ) was collected from a Millipore MilliQ system.

7.3.2. Electrospinning

For electrospinning, 10 % w/v PCL or 15% w/v PLGA solutions were prepared by dissolving the polymers in a mixture of DMF and DCM (1: 4 v/v). All the experiments were conducted at a temperature of 20-24 °C, with a humidity of approx. 40 %.

7.3.2.1. Preparation of PCL or PLGA Fibres via Single-Fluid Electrospinning

After a series of preliminary optimization experiments, single-fluid electrospinning was developed to produce PCL, PLGA1 and PLGA2 fibres. The formulations and electrospinning parameters are detailed in Table 7-1. The spinneret was connected to the positive electrode of the power supply using an alligator clip and fibres were collected on a flat plate aluminium collector attached to the grounded electrode. The working fluids were ejected using a flat-tipped needle with an inner diameter of 0.84 mm. The flow rate was 2.0 mL/h. LDH-MTX was synthesized according to the protocol in Section 2.1.2. A magnetic stirrer bead was placed inside the syringe and used to ensure that the LDH-MTX nanocomposites were well-dispersed in the fluids throughout the electrospinning process, via stirring 50 rpm.
Table 7-1 Details of the formulations of PCL and PLGA generated by single-fluid electrospinning

<table>
<thead>
<tr>
<th>Fibres</th>
<th>Polymer (w/v)*</th>
<th>Voltage (kV)</th>
<th>Distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 1</td>
<td>15% PLGA 150 kDa, 75/25, carboxylic group terminated</td>
<td>16.0</td>
<td>8</td>
</tr>
<tr>
<td>PLGA 2</td>
<td>15% PLGA 82 kDa, 50/50, ester group terminated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL</td>
<td>10% PCL 80 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL-LDH</td>
<td>10% PCL 80 kDa and 40 w/w % LDH-MTXb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a: concentrations based on the solvent volume. b: ratio between the weight of LDH-MTX to dry PCL polymer.

7.3.2.2. Preparation of PCL or PLGA Fibres Loaded with SPIONs and LDH-MTX via Co-Axial Electrospinning

For co-axial electrospinning, the core and shell solutions were loaded into two syringes fitted onto a co-axial needle with inner diameters of 1.37 mm (shell) and 0.69 mm (core). All the experiments were conducted at a temperature of 20-24 °C, with a humidity of approx. 40 %. To produce LDH-MTX loaded fibres, the core fluid was prepared by adding the LDH to a 10% w/v PCL solution, with an LDH concentration of 40 % w/w with respect to the dry PCL weight. A magnetic stirrer bead was placed inside the syringe and used to ensure that the LDH-MTX nanocomposites were well-dispersed in the core fluids throughout the electrospinning process, via stirring 50 rpm. The spinneret was connected to the positive electrode of the power supply (fangsi-220, Dongwen High Voltage Co., Ltd. China) using an alligator clip, and fibres were collected on a flat plate aluminium collector attached to the grounded electrode, with a working distance of 8 cm. Full details of the spinning process are given in Table 7-2.

Table 7-2 The electrospinning parameters used to prepare core/shell fibres

<table>
<thead>
<tr>
<th>Fibre</th>
<th>Shell (w/v)*</th>
<th>Flow rate</th>
<th>Electrostatic field</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-LDH@PCL</td>
<td>10% PCL</td>
<td>2.0 mL/h (shell)</td>
<td>2.0 kV/cm</td>
</tr>
<tr>
<td>PCL-LDH@PLGA1</td>
<td>15% PLGA-1</td>
<td>0.6 mL/h (core)</td>
<td></td>
</tr>
<tr>
<td>PCL-LDH@PLGA2</td>
<td>15% PLGA-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a: concentrations based on the solvent volume.
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7.3.3. Preparation of Fibres Loaded with Gd(DTPA) or SPIONs

7.3.3.1. PDA Coating

About 30 mg of fibres (3 x 3 cm) were immersed in 30 mL of dopamine hydrochloride solution (1 mg/mL, dissolved in 10 mmol/L Tris–HCl at pH 8.5). 2 mL of NaIO₄ (10 mg/mL) was slowly added at room temperature. The resultant PDA coated fibres were collected, washed with deionized (DI) water three times and dried under vacuum.

7.3.3.2. Gd(DTPA) or SPIONs Loading

To load Gd(DTPA) or SPIONs, the PDA coated fibres were resuspended in 30 mL of 10 mM Tris–HCl (pH 8.5) containing dopamine hydrochloride (1 mg/mL) and PVP-SPIONs or Gd(DTPA) (2 mg/mL). The suspension was shaken at room temperature (50 rpm) for 1 h. Fibres loaded with Gd(DTPA) or SPIONs were obtained by washing with DI water three times and drying under vacuum.

7.3.4. Fibre Characterization

Morphology

The morphology of the fibres was characterised using SEM and TEM, in accordance with the protocols given in Section 2.4.1

Physicochemical Characterisation

The fibres were characterised using XRD, DSC, TGA and FTIR as described in Section 2.4.2

7.3.5. In vitro Dissolution Studies

7.3.5.1. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was performed using a Shimadzu LC-20AT instrument with a UV-vis detector (SPD-10A). Samples were filtered through a PVDF-type syringe filter (0.22 µm) before analysis. A reverse-phase C18 column (150 x 4.6 mm, 5 µm, ODS, Platisil, China) was used. The mobile phase (acetonitrile and
phosphate buffer (pH 6.0) at a ratio of 8:92 v/v) was filtered (as above) and degassed prior to use. The flow rate of the mobile phase was 1.4 mL/min.

7.3.5.2. **Loading Capacity (LC) and Encapsulation Efficiency (EE)**

The LC and EE % were measured as described in Section 2.5.3.2.

7.3.5.3. **MTX Release Studies**

The MTX release study was undertaken using a 25 mL suspension of fibres (~ 0.5 mg/mL). Samples (n=3) were dispersed in 5 mL of the desired buffer (pH 7.4 phosphate buffered saline (PBS) or pH 5.0 acetate buffer) and transferred into a dialysis bag (MWCO=3500 Da). The bag was submerged into 20 mL of the respective buffer and stirred at 37 °C (50 rpm).

In the GSH-triggered release experiments, samples (n=6) were immersed in 5 mL of PBS buffer (pH 7.4) and transferred into a dialysis bag (MWCO=3500 Da), which was in turn submerged into 20 mL of PBS (pH 7.4) and stirred at 37 °C (50 rpm). After a 2 hour incubation, half the samples (n=3) were transferred to PBS supplemented with 20 mM GSH. In all cases, 0.5 mL aliquots were periodically withdrawn from the dissolution medium and filtered through PVDF-type syringe filters (0.22 µm) for HPLC analysis. To maintain a constant volume, 0.5 mL of fresh pre-heated buffer was added to the dissolution vessel after the removal of each aliquot. Experiments were performed in triplicate and the results are reported as mean ± S.D.

7.3.6. **In vitro Cell Studies**

The detailed protocol for cell culture, PrestoBlue™ cell viability assay and crystal violet staining are depicted in Section 2.6.

7.3.6.1. **Cell Viability Studies**

For viability assays, cells were pre-grown in 24 well plates (ThermoScientific, UK). 5 × 10^4 cells in 1 mL of medium were seeded into each well and cultured for 24 h. The medium was aspirated and 1 mL of pre-heated DMEM-HG containing various concentrations of the formulations or MTX (corresponding to free MTX concentrations of 50 ng/mL) added. The cells were incubated at 37 °C, under 5 % CO₂. After 24 h, cell
viability was determined with a PrestoBlue™ cell viability assay (ThermoFisher, UK) followed by crystal violet analysis on the same plate.

7.3.6.2. Reductive Response Studies

Reduced glutathione (GSH, 5 mM) was added to A549 or Caco-2 cells treated with PCL-LDH@PCL or PDA coated PCL-LDH@PCL fibres (corresponding to free MTX concentrations of 50 ng/mL). The above procedures were then followed to determine viability.

7.3.7. Relaxivity Measurement

Proton relaxivity ($r_{1,2}$) was measured as described in Section 2.4.3.2. To monitor changes in proton relaxivity with pH and temperature, a dispersion of approximately 20 mg of each formulation in 10 mL of a 0.1 % (w/v) aqueous xanthan gum solution was incubated at 37 °C, 50 rpm. At selected time points, 1.5 mL aliquots were transferred into a 10 mm-diameter NMR tube in order to measure the longitudinal or transverse relaxation time ($T_1$ and $T_2$). The aliquots were then returned to the vessel for further incubation. After relaxivity monitoring, the remaining fibres were recovered and digested using hot HNO$_3$ digestion, and then diluted to 10 mL with deionized water. The total [Gd]/[Fe] concentrations (mM) comprise the sum of the metal concentrations in the medium and fibres, which were quantified on an Agilent 7500cx inductively coupled plasma mass spectrometer (ICP-MS). All experiments were performed in triplicate and the results are reported as mean ± S.D.

7.3.8. MRI

To give good resolution in a clinical MRI scanner, the fibre mat (~1.2 cm x 1.5 cm) and immersed in a 15 mL centrifuge tube with 0.1 % (w/v) aqueous xanthan gum solution, as shown in Figure 7-4A.
Figure 7-4 Illustration of MRI scanning specimens (A); specimens were fixed in centrifuge rack before scanning.

MRI scanning was conducted by Dr Fenglei Zhou and Dr Lorna Smith from the Centre for Medical Image Computing, UCL. Samples were fixed into a centrifuge rack before scanning (Figure 7-4B). $T_1$ MRI scans were conducted in two directions (horizontal and vertical, Figure 7-4A) and $T_2$ scans in the vertical direction (Figure 7-4A).

$T_1$ weighted and $T_2$ weighted sequences were performed on the samples using a clinical MRI scanner (3T Ingenia, Philips, Best, Netherlands). Both sequences were acquired in the transverse plane with resolution $1\text{mm} \times 1\text{mm}$, $2\text{mm}$ slice thickness, reconstructed resolution was $0.3\text{mm} \times 0.3\text{mm}$, $2\text{mm}$ slice thickness. Parameters for the $T_1$ and $T_2$ weighted sequence were given in Table 7-3.
Table 7-3 Parameters for the $T_1$ and $T_2$-weighted sequence

<table>
<thead>
<tr>
<th></th>
<th>$T_2$</th>
<th>$T_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>TSE</td>
<td>TSE</td>
</tr>
<tr>
<td>TR (ms)</td>
<td>4971</td>
<td>540</td>
</tr>
<tr>
<td>TE (ms)</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>Flip angle (degrees)</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>TSE factor</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Acq resolution (mm)</td>
<td>1 x 1 x 2</td>
<td>1 x 1 x 2</td>
</tr>
<tr>
<td>Recon resolution (mm)</td>
<td>0.3 x 0.3 x 2</td>
<td>0.3 x 0.3 x 2</td>
</tr>
<tr>
<td>FOV(mm)</td>
<td>160 x 160 x 70</td>
<td>150 x 221 x 46</td>
</tr>
<tr>
<td>Plane</td>
<td>transverse</td>
<td>transverse</td>
</tr>
</tbody>
</table>

7.4. Results

7.4.1. Electrospun Fibres

Prior to coaxial electrospinning, the biodegradable polymers [22] PCL or PLGA were firstly electrospun alone. Core-shell PCL or PLGA fibres were then produced to encapsulate the MTX loaded nanocomposite (LDH-MTX). The characterization of LDH-MTX can be found in Chapter 5.4.2.

7.4.1.1. Monolithic Fibres

We first optimized the electrospinning parameters for both the core and shell working fluids. The optimized shell fluid consisted of 10 w/v % PCL or 15 % w/v PLGA1/2 in a mixed solvent system of DMF and CH$_2$Cl$_2$ (1: 4 v/v). The optimized core fluid consisted of 10 w/v % PCL with 4% LDH-MTX in a mixed solvent system (1: 4 w/v).

The monolithic PCL fibres display cylindrical and smooth morphologies (Figure 7-5), with a mean size of 1.06 ± 0.44 μm.
The morphology of the PLGA fibres is distinct to that of PCL. Both PLGA1 and PLGA2 fibres have ribbon-like morphologies (Figure 7-6), with mean diameter of $0.65 \pm 0.31$, $0.54 \pm 0.39 \mu m$. A small proportion of beaded fibres can be found in the PLGA2 sample, which might due to the low molecular weight this polymer. This leads to a relatively low solution viscosity, and thus to a situation intermediate between electrospinning and spraying and the formation of beaded fibres [23, 24].

After loading LDH-MTX, the PCL fibres have relatively larger diameters ($1.16 \pm 0.51 \mu m$), less structural uniformity and more twisted configurations, as shown in Figure 7-7. Elemental mapping confirms the presence of LDH-MTX, which is roughly homogenous distributed inside the fibre.
Figure 7-7 SEM images (A, C), size distribution (B) and Mg/Al elemental mapping (D,E) of PCL-LDH.

7.4.1.2. Core-shell Fibres

The drug–LDH distribution in the coaxial fibres was explored using SEM-EDX and TEM analyses. The SEM images and elemental mapping results for Mg and Al of resultant PCL@PCL-LDH or PLGA@PCL-LDH fibre are presented in Figure 7-8 to 7-10. The fibres are largely smooth and cylindrical in morphology, but the PLGA1@PCL-LDH and PLGA2@PCL-LDH fibres (formulation names as detailed in Table 7-1) show evidence of solvent inclusion in the form of merged fibres. Mg and Al are distributed evenly throughout the fibres, indicating that the drug loaded LDH nanoparticles were well dispersed as a result of magnetic stirring. The average diameters of the PCL, PLGA1 and PLGA2@PCL-LDH fibres are $1.48 \pm 0.58$, $1.48 \pm 0.55$ and $1.23 \pm 0.54 \text{µm}$, respectively.
Figure 7-8 SEM images (A, C), size distribution (B) and Mg/Al elemental mapping (D, E) of PCL-LDH@PCL.

Figure 7-9 SEM images (A, C), size distribution (B) and Mg/Al elemental mapping (D, E) of PCL-LDH@PLGA1.
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Figure 7-10 SEM images (A, C), size distribution (B) and Mg/Al elemental mapping (D, E) of PCL-LDH@PLGA2.

To study the internal structure of the fibres, TEM images were obtained (Figure 7-11). The core-shell structures of the fibres are clearly visible, confirming that coaxial spinning had been successfully performed.

Figure 7-11 TEM images of PCL-LDH@PCL (A and B), PCL-LDH@ PLGA1 (C and D) and PCL-LDH@ PLGA2 (E and F) (scale bar: 1 μm).
7.4.1. Preparation of Fibres@Gd(DTPA) or SPIONs/PDA

The PVP-SPIONs used here were previously prepared as detailed in Section 2.1.1, and characterised in Section 3.4.1. The preparation of Gd(DTPA) or SPIONs/PDA loaded fibres is illustrated in Scheme 7-1B, and involves two steps. In the first step, PDA coated fibres were prepared. Subsequently, in step 2 MRI contrast agents (Gd(DTPA) or SPIONs) were incorporated onto the surface of the PDA-coated fibres. This led to a family of fibres as listed in Table 7-3.

Table 7-4 The different fibres@Gd(DTPA) or SPIONs/PDA systems prepared

<table>
<thead>
<tr>
<th>Formulation ID</th>
<th>Fibre</th>
<th>Post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL@P</td>
<td>Blank PCL fibres</td>
<td>PDA coating (Step 1)</td>
</tr>
<tr>
<td>P-PCL</td>
<td>PCL-LDH@PCL</td>
<td></td>
</tr>
<tr>
<td>P-PLGA1</td>
<td>PCL-LDH@PLGA1</td>
<td></td>
</tr>
<tr>
<td>P-PLGA2</td>
<td>PCL-LDH@PLGA2</td>
<td></td>
</tr>
<tr>
<td>S-P-PCL</td>
<td>PCL-LDH@PCL</td>
<td>PDA coating and SPIONs loading (Step 1 and 2)</td>
</tr>
<tr>
<td>S-P-PLGA1</td>
<td>PCL-LDH@PLGA1</td>
<td></td>
</tr>
<tr>
<td>S-P-PLGA2</td>
<td>PCL-LDH@PLGA2</td>
<td></td>
</tr>
<tr>
<td>G-P-PCL</td>
<td>PCL-LDH@PCL</td>
<td>PDA coating and Gd (DTPA) loading (Step 1 and 2)</td>
</tr>
<tr>
<td>G-P-PLGA1</td>
<td>PCL-LDH@PLGA1</td>
<td></td>
</tr>
<tr>
<td>G-P-PLGA2</td>
<td>PCL-LDH@PLGA2</td>
<td></td>
</tr>
</tbody>
</table>

7.4.1.1. PDA Coated Fibres

The as-prepared fibres were immersed in a dopamine solution to deposit polydopamine on their surfaces. The self-polymerization of dopamine in solution can lead to polydopamine nanoclusters being attached onto almost any substrate (both hydrophobic and hydrophilic). To accelerate the polymerization, an oxidant (NaIO₄) was added.

Morphology

PCL@P

Firstly, blank PCL fibres coated with PDA were produced. SEM images (Figure 7-12) revealed that some areas of the fibre surfaces become darker (indicative of PDA coating) and a number of PDA particles can be seen. This proves a successful PDA coating.
Figure 7-12 SEM images (A, B) of PCL@P fibres.

PDA Coated Core-shell Formulations

The morphology of PDA coated PCL or PCL-LDH@PLGA composites was explored using SEM-EDX analyses. The PDA decorated PCL or PLGA@PCL-LDH fibres maintained similar morphology to the pristine fibres, with a generally homogeneous PDA coating on the fibre surface. This can be observed from SEM images (Figure 7-13 to 7-15). Although a few large particles of PDA can be observed, N (present only in PDA) is seen to be evenly distributed in EDX. The fibre size did not significantly increase after PDA coating, with diameters of 1.56 ± 1.03, 1.54 ± 0.68 and 1.20 ± 0.68 µm for PDA coated PCL-LDH@PCL (P-PCL), PDA coated PCL-LDH@PLGA1 (P-PLGA1) and PCL-LDH@PLGA2 (P-PLGA2), respectively (see Table 7-3 for details of sample nomenclature).
Figure 7-13 SEM images (A, C), size distribution (B) and Mg, Al and N elemental mapping (D, E, F, respectively) of P-PCL.

Figure 7-14 SEM images (A, C), size distribution (B) and Mg, Al and N elemental mapping (D, E, F, respectively) of P-PLGA1.
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Figure 7-15 SEM images (A,C), size distribution (B) and Mg, Al and N elemental mapping (D, E, F, respectively) of P-PLGA2.

7.4.1.2.  Gd(DTPA) or SPIONs Loaded Core-shell Fibres

Subsequently, MRI contrast agents (Gd(DTPA) or SPIONs) were incorporated onto the surface of the PDA-coated fibres. The hydroxyl groups and nitrogen containing groups in PDA can undergo hydrogen bonding, promoting interfacial interactions between the contrast agents and the fibres. Simultaneously, PDA was deposited onto the fibre surface to fix the contrast agents in place.

SEM images show that the thickness of the PDA layer was increased and greater numbers of PDA clusters were created after the introduction of MRI contrast agents and second stage of PDA coating, greatly increasing the fibre surface roughness (Figure 7-16, 7-17 and Figure A1-4). The fibre size slightly increases compared to the pristine fibres, confirming the success of surface decoration, with a diameter of 1.41 ± 0.69, 1.45 ± 0.60, and 1.25 ± 0.64 µm for SPIONs loading P-PCL (S-P-PCL), P-PLGA1 (S-P-PLGA1), P-PLGA2 (S-P-PLGA2), and 1.44 ± 0.74, 1.51 ± 0.56, 1.30 ± 0.56 µm for
Gd(DTPA) loading P-PCL (G-P-PCL), P-PLGA1 (G-P-PLGA1) and P-PLGA2 (G-P-PLGA2), respectively.

Figure 7.16 SEM images (A, C), size distribution (B) and Gd (D) elemental mapping of G-P-PCL.

Figure 7.17 SEM images (A, C), size distribution (B) and Fe (D) elemental mapping of S-P-PCL.
Elemental mapping results suggest the MRI contrast agents were successfully loaded on the fibre surface. For Gd(DTPA) loaded fibres, the Gd was uniformly distributed, while for the SPION loaded fibres, the Fe intensity is higher in the regions of large PDA clusters. This might be a result of the SPIONs aggregating with PDA.

7.4.2. Fibre Characterization

7.4.2.1. FTIR

Core-shell Fibres

Figure 7-18 displays the FTIR spectra of the core-shell fibres and raw materials. The spectra of core-shell fibres appear identical to that of the shell polymer, and peaks from LDH-MTX cannot be identified. This might because LDH nanoparticles were localized inside the fibre core and the loading amount is low (theoretical LC, 8.5 % for PCL-LDH@PCL, and 6.5 % for PCL-LDH@PLGA1/2).
Figure 7-18 FTIR spectra of raw materials, PCL-LDH@PCL, PCL-LDH@PLGA1 and PCL-LDH-PLGA2 fibres (for sample nomenclature see Table 7-3).

Fibres@ Gd(DTPA) or SPIONs/PDA

The IR spectra of fibres after PDA post-treatment (Figure 7-19) confirm the presence of Gd(DTPA) or the SPIONs in the formulations. In the spectra of Gd(DTPA)-containing systems the characteristic Gd(DTPA) carbonyl band at 1580 cm$^{-1}$ can clearly be seen, somewhat shifted to around 1600 cm$^{-1}$. For SPION loaded fibres, the Fe-O peaks around 540 cm$^{-1}$ can be identified.
Figure 7-19 FTIR spectra of (A) Gd(DTPA), SPIONs, PCL-LDH@PCL, S-P-PCL and G-P-PCL fibres; (B) PCL-LDH@PLGA1, S-P-PLGA1 and G-P-LGA1 fibres; (C) PCL-LDH@PLGA2, S-P-PLGA2 and G-P-LGA2 fibres (for sample nomenclature see Table 7-3).

7.4.2.2. XRD

As shown in Figure 7-20, all the fibres display the major reflections from semi-crystalline PCL in XRD (at 20 of 21° and 23.5°) [25, 26]. Reflections of SPIONs can be found in the XRD patterns of the SPION-loaded fibres, suggesting their successful incorporation. The reflections of LDH-MTX were not observed, which might because they have low intensity and thus were obscured by other components of the system.
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Figure 7.20 XRD patterns of the raw materials and (A) PCL-LDH@PCL, P-PCL, G-P-PCL and S-P-PCL; (B) PCL-LDH@PLGA1, P-PLGA1, G-P-PLGA1 and S-P-PLGA1; and (C) PCL-LDH@PLGA2, P-PLGA2, G-P-PLGA2 and S-P-PLGA2 (for sample nomenclature see Table 7.3).

7.4.2.3. XPS Spectra

XPS was employed to further analyse the chemical composition of the fibres. In the following sections, XPS survey scans and detailed spectra of PCL-LDH@PCL-based materials are presented as exemplar systems. Spectra describing the PCL-LDH@PLGA-based materials can be found in Appendix 2.
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Core-Shell Fibres

For electrospun PCL or PLGA/PCL fibres (Figure 7-21, Figure A2-1), peaks arising from C and O are identified. The triplet C 1s peak of PCL-LDH@PCL, PCL-LDH@PLGA1 and PCL-LDH@PLGA2 can be deconvoluted into three components, comprising O=C-O (288.8 eV), C-O (286.7 eV) and C-C/H (284.7 eV) groups. Related peaks can be found in the high resolution O spectra. The decomposition of the O 1s envelope revealed the presence of two types of oxygen: O=C at 531.8 eV and O-C at 533.1 eV. These observations are all consistent with the expected polymer fibre surface composition [27].

![XPS spectra](image)

Figure 7-21 Survey scan XPS spectrum (A), and the C 1s (B), and O 1s (C) regions of PCL-LDH@PCL.

PDA Coated Electrospun Fibres

Survey and high-resolution spectra, for P-PCL, P-PLGA1 and P-PLGA2 are displayed in Figure 7-22 and Figure A2-2 & 3, respectively.
After PDA decoration of the core-shell fibres, the presence of N in the XPS spectra can be seen, suggesting the successful deposition of PDA on the fibre surface. Fitting of the N 1s core-level spectra resulted in three peaks at 398.3, 399.7 and 401.5 eV, which were respectively attributed to N\(^+\) species (tertiary/aromatic amine functionalities), C-N (secondary amine functionalities) and R-NH\(_2\) (primary amine functionalities). The C 1s region is fitted into four main components assigned to C-C/C-H (284.7 eV), C-O/C-N (286.1 eV), C=O (288.7 eV) and \(\pi-\pi^*\) species (291.5 eV). In the O 1s spectra, an evident peak from H\(_2\)O at 533.5 eV is observed in some samples, which is assigned to water molecules adsorbed on the fibre surface. All results reflected changes in atomic composition of the fibre surface, which become more hydrophilic as a result of with polar groups and can be used for further functionalization.
Fibres@ Gd(DTPA) or SPIONs/PDA

The N, C and O spectra of the Gd(DTPA) or SPION loaded fibres are similar to those of the PDA modified fibres (see Figure 7-23 and 24, Figure A2-4 to 2-7), indicating that PDA decoration is not impacted by coating with contrast agent species. The presence of Fe and Gd can clearly be seen in the MRI contrast agent-loaded materials.

Figure 7-23 Survey scans XPS spectrum and the C 1s, O 1s, N 1s and Fe 2p regions of S-P-PCL.

Figure 7-24 Survey scans XPS spectrum, C 1s, O 1s, N 1s and Gd 3d regions of G-P-PCL.
For efficient MRI, paramagnetic metal ions should closely interact with water protons. Some of the hydrophobic systems encapsulating MRI contrast agents suffer from low relaxivity due to poor accessibility to water. In the fibre systems produced here, the MRI contrast agents were incorporated onto the surface PDA layer, which should allow easy access to water. However, the surface hydrophilicity could significantly affect the MRI properties of the materials.

The water contact angle was thus determined. Firstly, the water contact angles of monolithic PLGA, PCL and PCL-LDH fibres were determined and the results are shown in Figure 7-25 and Table 7-4. Clearly, both the PLGA and PCL fibres are hydrophobic, and loading of LDH does affect the hydrophobicity of the fibres.

Figure 7-25. Photographs exhibiting the water contact angle at 3 s of (A) PCL, (B) PLGA1, (C) PLGA2 and (D) PCL-LDH fibres.
Table 7-5 Contact angles of monolithic fibres at 3 s.

<table>
<thead>
<tr>
<th>Fibres</th>
<th>Contact angle (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA1</td>
<td>136.0 ± 0.7</td>
</tr>
<tr>
<td>PLGA2</td>
<td>138.8 ± 1.1</td>
</tr>
<tr>
<td>PCL</td>
<td>139.0 ± 1.6</td>
</tr>
<tr>
<td>PCL-LDH</td>
<td>138.9 ± 0.6</td>
</tr>
</tbody>
</table>

PDA is seen to markedly increase the hydrophilicity of the electrospun fibres. As shown in Figure 7-26 and Table 7-5, the water contact angles (CA) of the pristine core/shell fibres (PCL-LDH@PCL, PCL-LDH@PLGA1 and PCL-LDH@PLGA2) are 141.7 ± 0.8°, 139.5 ± 4.1° and 134.2 ± 1.6°, respectively. The water droplet retains the same shape and remains almost unchanged on the fibre surface even after 30s. In contrast, the CAs of PDA coated fibres (P-PCL, P-PLGA1 and P-PLGA2) drop from an initial 86.6 ± 6.2, 82.4 ± 4.4 and 85.3 ± 5.8 ° to around 30° at 3 s and declines to 0 ° within 30 s. Similar trends can be observed with the Gd(DTPA) or SPIONs loaded fibres. This superhydrophilicity originates from the presence of abundant polar groups in the surface PDA molecules, and the increased surface roughness after PDA coating. For efficient MRI contrast, paramagnetic metal ions should closely interact with water protons. This should result in a high MRI contrast ability and low toxicity.
Figure 7-26 Photographs exhibiting the dynamic change of water droplets on (A) PCL-LDH@PCL, (B) PCL-LDH@PLGA1 and (C) PCL-LDH@PLGA2 before and after post-fabrication treatments (as labelled); (D) the water CAs of the different fibres as a function of time.
7.4.3. In vitro Dissolution Studies

7.4.3.1. Drug Loading

HPLC

To determine drug loading, the fibres were fully dissolved and analysed using HPLC. A calibration curve was first constructed (Figure 7-27). Representative HPLC spectra of different samples and a standard MTX solution are given in Figure 7-28. MTX can be well separated from the other components of formulations.
Figure 7-27  MTX calibration curve based on HPLC analysis at $\lambda_{\text{max}}$ of 303 nm (Data obtained from three independent measurements as mean ± S.D.).

Figure 7-28  Representative HPLC chromatograms recorded at $\lambda_{\text{max}}$ of 303 nm.
LC and EE

The LC of the different fibres are summarized in Table 7-6. The drug loading is found to be 2.86 ± 0.37, 2.18 ± 0.25 and 2.04 ± 0.23 % w/w for PCL-LDH@PCL, PCL-LDH@PLGA1 and PCL-LDH@PLGA2 fibres, with the encapsulation efficiencies 74.2 ± 9.6, 75.6 ± 8.7 and 70.8 ± 8.0 %. The results indicate that the majority of drug in the feedstock is carried through into the fibres during the electrospinning process. Given the fact that MTX was loaded in the LDHs, it is thought that the losses observed arise from some LDH particles sedimenting at the bottom of the syringe. As would be expected, the MTX loading capacity decreases after PDA modification and contrast agent loading, lying in the range of 1.54 to 2.01 % (Table 7-6).

<table>
<thead>
<tr>
<th>Fibres</th>
<th>Loading capacity (n=3)</th>
<th>Encapsulation efficiency (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-LDH@PCL</td>
<td>2.86 ± 0.37 %</td>
<td>74.2 ± 9.6 %</td>
</tr>
<tr>
<td>PCL-LDH@PLGA1</td>
<td>2.18 ± 0.25 %</td>
<td>75.6 ± 8.7 %</td>
</tr>
<tr>
<td>PCL-LDH@PLGA2</td>
<td>2.04 ± 0.23 %</td>
<td>70.8 ± 8.0 %</td>
</tr>
<tr>
<td>P-PCL</td>
<td>2.01 ± 0.40 %</td>
<td>-</td>
</tr>
<tr>
<td>P-PLGA1</td>
<td>1.85 ± 0.36 %</td>
<td>-</td>
</tr>
<tr>
<td>P-PLGA2</td>
<td>1.77 ± 0.29 %</td>
<td>-</td>
</tr>
<tr>
<td>G-P-PCL</td>
<td>1.95 ± 0.34 %</td>
<td>-</td>
</tr>
<tr>
<td>G-P-PLGA1</td>
<td>1.54 ± 0.25 %</td>
<td>-</td>
</tr>
<tr>
<td>G-P-PLGA2</td>
<td>1.64 ± 0.28 %</td>
<td>-</td>
</tr>
<tr>
<td>S-P-PCL</td>
<td>2.03 ± 0.32 %</td>
<td>-</td>
</tr>
<tr>
<td>S-P-PLGA1</td>
<td>1.66 ± 0.31 %</td>
<td>-</td>
</tr>
<tr>
<td>S-P-PLGA2</td>
<td>1.59 ± 0.18 %</td>
<td>-</td>
</tr>
</tbody>
</table>

Low dosage (~25-50 mg/per week) MTX has been proven to be an effective adjuvant chemotherapy to treat patients with cancer, for instance for those with heavily pretreated breast cancer, or metastatic head and neck cancer [28-30]. Based on clinical biodistribution studies [31, 32], this dosage results in a MTX concentration of approximately 0.5 ~ 1 µg/kg tumour per day. Hence, around 45 ~ 90 µg/kg tumour of MTX is required for localized treatment in the target site for 90 days. To deliver this,
around 2 ~ 5 mg of the fibre formulations would be required. Hence, the fibres possess a reasonable drug loading amount and could be practicably applied in the clinic for localised MTX treatment.

### 7.4.3.2. MTX Release Studies

**Release Profile**

**Figure 7-29A, C, E** present the cumulative MTX release profiles over 18 days from the PCL-LDH@PCL, PCL-LDH@PLGA1 and PCL-LDH@PLGA2 systems before and after PDA modification, while **Figure 7-29B, D, F** contain enlargements of the first 24 h. From the enlargement of the first 24 h of release, it can be observed that there is fast release in the initial period of the experiment (approximately 2 hours) for both monolithic and core-shell fibres without modification followed by a slow and sustained release. The first stage of release comes from free MTX molecules which have deintercalated from the LDH particles during the electrospinning process. The second stage of release begin after two hours, mainly caused by the MTX escaping from the inside of the polymer matrix.

Compared to PCL fibres loaded with LDH-MTX via monoaxial electrospinning without a shell (PCL-LDH, **Figure 7-29A**), core@shell fibres can suppress the initial burst release of MTX in stage 1, while the sustained release in following stage was also slightly restrained. The PCL-LDH fibres rapidly released ≈ 60 % of its MTX payload in the first 7 h in PBS (pH 7.4), around 40 % was released from core-shell fibres. MTX release was further suppressed after PDA modification, decreasing to around 20% of total MTX for MRI contrast agents loaded fibres. This is probably caused by the diffusion barrier presented by the PDA layer.
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Figure 7-29 Plots showing the release of MTX from (A) PCL-LDH and PCL-LDH@PCL, (C) PCL-LDH@PLGA1 and (E) PCL-LDH@PLGA2 fibres over 432 hours with enlargements of the first 24 h for (B) PCL-LDH and PCL-LDH@PCL, (C) PCL-LDH@PLGA1 and (E) PCL-LDH@PLGA2 fibres. Data obtained by HPLC analysis (λ=303 nm) and given from three independent experiments as mean ± S.D.

The cumulative MTX release profile of PCL-LDH@PLGA hybrid systems (37 °C, pH 7.4) are presented in Figure 7-29C-F. Similar trends can be seen to PCL-LDH@PLGA systems. Clearly, PDA modification can significantly suppress the initial burst of release and prolong the release period of MTX.
A large initial burst release of MTX has been reported to cause sporadic toxicity among experimental animals [33]. The PDA decorated fibres prepared here have low initial burst, and near zero order drug release (Figure 7-30) over a prolonged period of time. This could help to maintain a constant MTX concentration in the tumour.

**Fibre Morphology after Dissolution Studies**

The morphology of the fibres after dissolution was investigated by SEM. Images of PCL-LDH@PCL/PLGA (Figure 7-31) showed that the fibrous structures were maintained after 18 days but some fibres were broken, suggesting the degradation of PCL and PLGA. PCL-LDH@PLGA1 and PLGA2 systems also present as broken fibres after the drug release study. The P-PCL and P-PLGA1 fibres displayed roughly intact fibre structures with clear edges after the dissolution experiment. But in the SEM images
of P-PLGA2, broken fibres with clear edges can be observed. This can be related the smaller molecular weight and the more hydrophilic glycolate content in PLGA2 (lactide:glycolide: 50:50), leading to a faster degradation rate in aqueous PBS. Clearly, PDA modification can significantly suppress the initial burst of release and prolong the release period of MTX.

It can be seen that PDA clusters remained on the surface of the fibres even after 18 days. The relatively intact fibre morphology indicates that PDA decoration might inhibit the degradation of PCL and PLGA and also might contribute to a prolonged MTX release.

![Figure 7-31 SEM images of (A) PCL-LDH@PCL, (B) PCL-LDH@PLGA1, (C) PCL-LDH@PLGA1, (D) P-PCL, (E) P-PLGA1, and (F) P-PLGA2 fibre samples after dissolution test for 432h.](image)

**XPS Analysis**

Quantitative XPS analysis was performed to study the composition change on fibre surface after dissolution test. Representative survey spectra for PCL-LDH@PCL are given in Figure 7-32, and for PCL-LDH@PLGA1/2 fibres in Figure A2-8. The results suggest the decomposition of PCL. As reported in [34, 35], the degradation of PCL are dominantly driven by random chain scission and can proceed through both acid and base catalyzed ester hydrolysis [34, 35]. This is proved by the broader peaks of C-O and C=O become as shown Figure 7-32B, C.
Figure 7-32 Survey scan and detailed C 1s, O 1s spectra of PCL-LDH@PCL after dissolution for 432 h.

Representative spectra for P-PCL are shown in Figure A1-9. Generally, XPS signals of C, N, and O were measurable in all PDA decorated fibres, suggesting the presence of PDA after 432 h of the dissolution test. This is consistent with the SEM images in Figure 7-31.

7.4.3.3. Stimuli-Responsive Release Studies

PDA-based drug delivery systems can be responsive to stimuli including pH [36, 37] and reductive agents such as GSH [38]. The release of MTX under different conditions was thus investigated.

pH

The release of MTX was investigated at 37 °C and pH 5.0, which was used to mimic the acidic microenvironments in tumours. As shown in Figure 7-33A-C, cumulative release of 58.6, 54.6 and 66.1 % of the MTX loading was reached for P-PCL, P-PLGA1 and P-PLGA2 at pH 5.0, greater than the values seen pH 7.4. This can be associated with PDA being detached from the fibre surface in acidic pH [36, 37], and might also result from dissolution of the LDH particles [39, 40], leading to a faster release. This pH-dependent release could be beneficial in localising release at a tumour site.
Figure 7.33 Plots showing MTX release from (A) P-PCL, (B) P-PLGA1 and (C) P-PLGA2 at pH 5.0 and 7.4 over 432 h; MTX release profiles of (D) P-PCL, (E) P-PLGA1 and (F) P-PLGA2 with 0 mM (red) and 20 mM (black) GSH stimulus in PBS, pH 7.4. Data obtained by HPLC analysis (λ=303 nm) and given from three independent experiments as mean ± S.D.

**GSH**

The polymerization of dopamine involves an oxidation process. It is reported that reductive GSH can reverse this polymerization. The effect of GSH on the release behaviour of MTX was investigated in PBS at pH 7.4. A distinct increase in MTX release was seen after the introduction of GSH (Figure 7.33D-F) for all the PDA coated fibres. This presumably arises because the GSH causes the breakdown of the PDA coating, and thus triggers the release of the drug [38]. After dissolution tests, the presence of the S 2p peak can be seen in XPS spectra (Figure 7.34), as indicative of interaction between GSH and PDA on the surface.
The pH and GSH-responsive release behaviour is beneficial for the construction of implantable systems designed to release a drug in the acidic tumour microenvironment with the presence of high GSH concentrations [41, 42]. The fibre systems can not only maintain a constant drug concentration at normal physiological pH, allowing them to suppress tumour recurrence, but also enable accelerated MTX release at mildly acidic and GSH-enriched microenvironment to treat potential recurrence.

Overall, the in vitro drug dissolution studies showed that the shell polymer (PCL or PLGA) did not markedly affect the release profiles, with all three systems performing similarly.

### 7.4.4. In Vitro Cell Studies

Two different cell lines, Caco-2 and A549 were treated with placebo PCL and PDA coated PCL (PCL@P) fibres, as well as the PCL-LDH@PCL based formulations.

#### 7.4.4.1. Cytotoxic Effects

For both cell lines, cell viability results (Figure 7-35A) confirmed that the PCL fibres are biocompatible and do not induce cytotoxicity (cell viability > 95 %). It has been reported that PDA materials can induced anti-proliferative effects in breast and colon tumour cells [43]. This is borne out here, where a decrease in viability is noted upon treatment with PDA-coated PCL (PCL@P) fibres.
The cytotoxicity of the LDH-loaded implants to Caco-2 or A549 cells was also investigated. Changes in cell shape and decrease in cell density, as characteristics of apoptosis were shown in cultures treated with placebo PCL and PDA coated PCL (PCL@P) fibres, as well as LDH-loaded implants, after 24 h (Figure 7-36). The corresponding cell viability data are given in Figure 7-37. Similar to placebo PDA@P (Figure 7-36B), LDH-loaded implants without PDA modification (Figure 7-35D) can induce cell death. However, both cell types displayed higher viability after exposure to the fibres than when treated with free MTX, since not all the drug was released from the fibres during the in vitro experiments. After PDA modification, the anti-proliferative effects were further enhanced, which is similar to that free MTX (Figure 7-36E and 37).
Figure 7-36 Morphological changes reflecting Caco-2 cell apoptosis with different formulations (scale bar: 100 μm).
Figure 7-37 Cytotoxicity data for Caco-2 and A549 cells treated with MTX and the different fibres. The untreated cells control is used as a baseline with a viability of 100%.

The cell viability evaluated by Presoblue™ were similar for PLGA1/2 based materials. PLGA1 and PLGA2 fibre are biocompatible (Figure A3-1). PCL-LDH@PLGA1/2 materials have moderate cytotoxicity to both cell lines, and the anti-proliferative effects are enhanced by PDA (Figure A3-2).

7.4.4.2. Cytotoxic Effects in GSH-Enriched Environments

Further studies were carried out using P-PCL as model formulation to investigate the effect of GSH on cytotoxicity. Cells in GSH-enriched media were treated with MTX, PCL-LDH@PCL or P-PCL (corresponding to free MTX concentrations of 50 ng/mL). The results show that the viability of cells is not affected by GSH (Figure 7-38). Both PCL-LDH@PCL and P-PCL showed toxicity to Caco-2 cells, but there was no statistically significant difference in viability between GSH-treated and untreated cells incubated with PCL-LDH@PCL. However, P-PCL displayed greater cytotoxicity to Caco-2 cells in the presence of GSH. Similar results can be found with A549 cells. The observations herein clearly indicate the effectiveness of GSH stimulus on triggering the release of MTX from PDA-coated fibres.
Figure 7-38 The cell viability data of PCL-LDH@PCL and P-PCL fibres in GSH-enriched media (*p<0.05, **p<0.01). Data were statistically evaluated by Student t-tests. The untreated cells control is used as a baseline with a viability of 100%.

7.4.5. MRI Relaxation Behaviour

7.4.5.1. Relaxivity

Since \textit{in vitro} drug dissolution and cell results reflected that the shell polymer (PCL or PLGA) did not markedly affect the release profiles and cell viability, the relaxivity was monitored as a function of time using G-P-PCL and S-P-PCL as example implants. Both systems were explored at different pHs (see Figure 7-39). For G-P-PCL, the $r_1$ value rapidly reached a plateau ($\sim$ 4.0 mM·s$^{-1}$) after 1 hour in all conditions, indicating that the contrast agents are quickly freed into the medium. For S-P-PCL, the $r_2$ increased slightly over 18 days. This might be due to the SPIONs being slowly released as a result of PDA degradation. No obvious differences can be observed in acidic or GSH-enriched conditions.
In Vitro MR Imaging

In situ MRI was further carried out as shown in Figure 7-4A. Figure 7-40A gives the $T_2$ weighed images in the vertical direction, while the $T_1$ weighed images in horizontal and vertical directions are described in Figure 7-40B and C, respectively. Compared to PCL-LDH@PCL and P-PCL, the SPIONs loaded fibres give a strong dark contrast in the fibre and surrounding areas in both $T_1$ and $T_2$-weighted images. This indicates that the SPIONs are retained on the fibre surface, while the Gd(DTPA) loaded formulation enhanced the positive contrast of the surrounding medium due to the release of the complex. From the MRI results, it seems that S-P-PCL can perform as a good theranostic platform to provide local contrast for implants, while G-P-PCL does not since Gd(DTPA) was rapidly released into the surrounding medium.
Figure 7-40 MRI scans of blank, PCL-LDH, P-PCL, S-P-PCL and G-P-PCL: A, $T_2$-weighted images, vertical; B, $T_1$-weighted images; horizontal; C, vertical $T_2$ images. The fibre implants are marked in red boxes.
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7.5. Conclusion

Polydopamine-coated polycaprolactone/poly(lactic-co-glycolic) acid nanofibres were developed as a potential implant to ensure safe and sustained release of the chemotherapeutic drug methotrexate (MTX). Fibres were prepared by co-axial electrospinning and loaded with MTX-layered double hydroxide (LDH) nanocomposites in the core, yielding organic–inorganic hybrids with a diameter ranging from 1.23 to 1.48 µm. After surface coating with polydopamine and MRI contrast agents (SPIONs or Gd(DTPA)), the hydrophilicity profoundly increased and the SPIONs and Gd(DTPA) were proven to be evenly distributed on the surface, providing high MRI contrast. In vitro drug release studies showed the polydopamine (PDA) coated fibres gave sustained release of MTX over 18 days, and that the release profile is responsive to conditions representative of the tumour microenvironment such as slightly acidic pH values or elevated concentrations of the reducing agent glutathione (GSH). In vitro studies with Caco-2 and A549 cells showed highly effective killing with the PDA coated formulations, which was further enhanced at higher levels of GSH. The fibres hence have the potential to act as an implantable drug-eluting platform for the sustained release of cytotoxic agents within a tumour site, providing a novel treatment option for post-operative cancer patients.

7.6. References


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8.1. Summary of Research Findings

For the development of effective personalized medicine, it is important to image the disease condition and monitor the therapeutic effect as a function of time. As a result, there is a need to develop novel approaches to precisely assess the progression of a disease and quantify drug delivery \textit{in vivo}. Real-time monitoring of therapeutic outcomes can help to track and tailor drug doses in a timely and accurate manner for patients, allowing adjustments of the applied dose in order to keep the concentration within the therapeutic window. Reliable \textit{in vivo} drug release information and assessment of disease progression can not only help to reduce side effects but also increase therapeutic efficacy.

A promising strategy to achieve this potential involves the development of therapy guided by magnetic resonance imaging (MRI). MRI has a high degree of soft tissue contrast, low invasiveness, and high depth of penetration and spatial resolution. It is one of the most widely-used non-invasive clinical imaging tools, producing detailed anatomical images whilst avoiding side effects such as trauma or X-ray radiation exposure. The major goal of this work was to develop novel theranostic systems for MRI-guided chemotherapy.

8.1.1. \textbf{pH-Responsive Nanocomposite Delivery Systems}

In Chapter 3, a new approach to non-invasive MRI monitoring of drug release from a carrier vehicle was developed, using pH-responsive Eudragit L100, L100-55 and S100 microparticles or fibres encapsulating superparamagnetic iron oxide nanoparticles (SPIONs) and carmofur (a drug used in the treatment of colon cancer). First, SPIONs were synthesized as negative MRI ($T_2$) contrast agents. These nanoparticles were stabilized by polyvinylpyrrolidone (PVP), providing a stable suspension which could be used for the fabrication of microparticles. The PVP stabilized SPIONs (PVP-SPIONs) are stable in ethanol for at least 3 hours.

Microparticles or nanofibres were then produced via EHDA. The S100 and L100 microparticles emerged as irregularly shaped pseudo-spherical particles with rough surfaces, the mean diameters of which are $863 \pm 333$ nm and $419 \pm 176$ nm. The Eudragit L100-55 particles exhibited a different shape, being rod shaped with a mean width of $290 \pm 117$ nm and length of $902 \pm 411$ nm. Fibres were found to be smooth and
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cylindrical with diameters of 645 ± 225 nm for L100 and 454 ± 133 nm for S100. The formulations exhibited pH responsive dissolution behaviour. Around the physiological pH range, clear pH-responsive proton relaxation rate changes due to matrix swelling/dissolution can be observed, but below the pH at which Eudragit is soluble $r_2$ remains very low. These dramatic changes in relaxivity demonstrate that pH-responsive dissolution results in SPION release. In vitro drug release studies showed the formulations gave rapid release of carmofur at physiological pHs (pH 6.5 and 7.4), and acid stability studies showed that the formulations can protect the SPIONs from digestion in acid environments, giving them potential for oral administration.

Exploration of the relationship between relaxivity and carmofur release suggests a linear correlation between the drug release and $r_2$ profile. Mathematical equations were developed to predict carmofur release in vitro, with very similar experimental and predicted release profiles obtained. Therefore, the formulations developed here have the potential to be used for non-invasive monitoring of drug release in vivo, and could ultimately result in reductions to off-target side effects from interventions such as chemotherapy.

8.1.2. Dual Responsive Systems

Most conventional chemotherapeutics have narrow therapeutic windows, and thus their delivery remains challenging and often raises safety and efficacy concerns. Theranostic platforms, with simultaneous encapsulation of therapeutic and diagnostic agents, have been proposed as next-generation formulations which can overcome this issue. In Chapter 4, core@shell formulations comprising a pH responsive Eudragit L100 shell embedded with superparamagnetic iron oxide nanoparticles (SPIONs), and a thermo-responsive poly(N-isopropylacrylamide) (PNIPAM)/ethyl cellulose core loaded with the model drug carmofur were generated as anti-cancer theranostics. By varying the weight ratio of core polymer to shell polymer, the morphology of the PNIPAM/ethyl cellulose@Eudragit L100 microspheres changed from concave microparticles to spherical particles. Smooth cylindrical fibres could also be generated.

All the formulations exist as amorphous solid dispersions of drug-in-polymer, with distinct core@shell architectures. The fibres have clear thermo-responsive drug release profiles, while no thermo-responsive properties can be seen with the particles. All the formulations can protect SPIONs from degradation in gastric fluids (pH ~ 1.5), and
around the physiological pH range the materials offer effective and pH-responsive relaxivity. The $r_2$ values also display clear linear relationships with drug release data, suggesting the potential of using MRI signals to track drug release in vivo. Examples of mathematical equations were established to track drug release in vitro, with very similar experimental and predicted release profiles obtained. Thus, the PNIPAM/ethyl cellulose@Eudragit formulations developed herein can potentially be applied as controlled release systems for oral delivery of MRI-guided chemotherapy.

8.1.3. Thermo-responsive Nano-in-micro Particles

Hyperthermia is an effective treatment for cancer, enabling an elevation of tumour temperature to promote other treatments such as chemotherapy or radiotherapy. In Chapter 5, nano-in-micro thermo-responsive microspheres were prepared as theranostic systems for anti-cancer hyperthermia. Firstly, layered double hydroxide (LDH) nanoparticles were synthesized and subsequently loaded with the chemotherapeutic agents methotrexate (MTX) or 5-fluorouracil (5FU). The drug-loaded LDH particles were then co-encapsulated with superparamagnetic iron oxide nanoparticles (SPIONs) into poly(acrylamide-co-acrylonitrile) microparticles via spray drying. The SPIONs are able to act as MRI contrast agents, thus resulting in potential theranostic formulations. Concave microparticles were observed by electron microscopy, and elemental mapping results suggest the LDH and SPION particles were homogeneously distributed inside the microparticles. In vitro dissolution tests showed that the drug was released over a prolonged period of time with the microspheres having distinct release curves at 37 and 43 °C, reflecting their thermosensitive properties. The microparticles were also found to have thermo-responsive relaxivity ($r_2$) over the temperature range 35 to 46 °C, and in vitro cell experiments revealed that the formulations permit synergistic hyperthermia-aided chemotherapy in cultured Caco-2 and A549 cells. Thus, the microparticles might have potential as smart stimuli-responsive theranostics for hyperthermia-aided chemotherapy.

8.1.4. Gadolinium Doped Layered Double Hydroxides

LDH materials are protective and controlled delivery platforms that have attracted a lot of attention in biomedical research. These materials can be easily doped with a number of other metal ions to obtain functional nanomaterials for MRI or computed tomography.
In Chapter 6, four different MgAl-LDH formulations doped with Gd$^{3+}$ were fabricated and fully characterised by X-ray diffraction, IR spectroscopy and relaxivity measurements. The successful doping of Gd$^{3+}$ was confirmed by XPS spectra showing characteristic peaks of Gd (4p). All the particles display good $r_1$ relaxivity and thus can potentially be used to develop MRI-based theranostics.

To further load chemotherapeutics, a Gd$^{3+}$ doped LDH obtained by the ‘bottom-up’ method was selected because it can be obtained in one-step with desirable relaxivity. Two cytotoxic drugs, 5FU and MTX, were intercalated via ion-exchange. A stability assessment revealed that the Gd doped LDHs were stable under physiological conditions, while in acidic conditions a small proportion of Gd was freed into the immersion medium. In vitro release studies displayed that both 5FU and MTX were rapidly released from the LDH carrier, in a manner slightly responsive to acidic pH. The longitudinal relaxivity of Gd-LDHs remains roughly stable during drug release over 24h, and was higher in acidic conditions. Hence, the drug-loaded Gd-LDH nanosheets produced could serve as pH-sensitive theranostic platforms for MRI-guided chemotherapy.

### 8.1.5. Polydopamine-coated Nanocomposite Theranostic Implants

Sustained and localized delivery of chemotherapeutics in postoperative cancer treatment leads to a radical improvement in prognosis and a much decreased risk of tumour recurrence. In Chapter 7, a series of polydopamine-coated polycaprolactone/poly(lactic-co-glycolic) acid nanofibers was developed, aiming to offer implantable platforms to ensure safe and sustained methotrexate (MTX) treatment. Fibres were prepared by co-axial electrospinning and encapsulated with MTX-LDH nanocomposites in the core, yielding organic–inorganic hybrids with a diameter ranging from 1.23 to 1.48 µm. To suppress the initial burst release and provide local MRI contrast, the fibres were further coated with polydopamine (PDA) and SPIONs or gadolinium-DTPA. The hydrophilicity profoundly increased and MRI contrast agents, SPIONs and gadolinium-DTPA were proven to be evenly distributed on the surface. In vitro MTX dissolution release studies showed the PDA coated fibres gave sustained release of MTX over 18 days, and that the release profile is responsive to conditions representative of the tumour microenvironment such as slightly acidic pH values or elevated concentrations of the
reducing agent glutathione (GSH). In vitro cell studies with Caco-2 and A549 cells showed highly effective killing with the PDA coated formulations, which was further enhanced at higher levels of GSH. The fibres hence have the potential to act as implantable drug-eluting systems for the sustained release of cytotoxic agents within a tumour site, providing a novel chemotherapy option for post-operative cancer patients.

### 8.1.6. Summary

Overall, form all formulations produced in this PhD thesis, we found that nanocomposites, such as SPIONs and layered double hydroxides, can be encapsulated in polymeric matrices, and used as a ‘nano-in-micro’ strategy to produce multifunctional theranostic platforms. With the aid of stimuli-responsive polymers, it is possible to obtain theranostics reflecting local environment changes (e.g. temperature, pH) via the MRI signal based on relaxometry profile.

Among the five theranostic systems developed with the aim of delivery of anti-cancer therapy guided by MRI, the UCST microparticles in Chapter 5 are produced via spray drying, a one-step and well-established technique that can be easily scaled up. The layered double hydroxide-based theranotic nanosheets synthesized using a ‘bottom-up’ method also can be facilely produced in a large scale. While other formulations are prepared via EHDA, which can also be scaled up and performed under GMP conditions.

The new knowledge gained in this thesis can be informative for further research in developing MRI-guided systems by incorporating small molecule chemotherapeutics and MRI contrast agents into polymeric matrices via facile techniques such as electrospinning, electrospraying and spray drying. The findings of this work will thus be very helpful to researchers seeking to produce novel theranostic platforms. In the longer term, the formulations developed have the possibility to provide impact in terms of improved diagnosis and therapy for cancer patients, and ultimately could contribute to human health and wellbeing.

### 8.2. Future Work

The studies reported in this thesis have focused on platform design, physicochemical characterization, and functional performance exploration in vitro. Further investigation
is needed to improve the functionality of the formulations and move them closer to the clinic, which lies in three categories.

### 8.2.1. MRI

The most immediate further studies involve recording $T_1$ and $T_2$ weighted images of the contrast agent loaded theranostics platforms. For the formulations prepared in Chapter 3 and 4, the fibres should be incubated with porcine colon at different pH (5.0-7.4). For formulations produced in Chapter 5, 6 and 7, *ex vivo* models e.g. patient-derived tumor organotypic spheroids, or explanted cancer tissue can be employed. Images should be taken using a clinical MRI scanner under different conditions (hyperthermia temperatures, tumour microenvironment). This will determine whether the $T_1/T_2$-weighted images of the colon or tumour are clear and suitable for diagnosis. The drug release should be recorded in parallel experiments to further validate their ability to using MRI signal to predict drug release.

### 8.2.2. Formulation Optimization

For the oral delivery dual-responsive systems developed in Chapter 4 a UCST polymer, instead of the LCST polymer PNIPAM, could be employed to vary the thermo-responsive properties. In such a system, drug release can be triggered using hyperthermia, rather than hypothermia, which can potentially further enhance the anti-cancer therapeutic efficacy of chemotherapy as discussed in Chapter 5. Since we found that the fibre structures work better as thermo-responsive platforms in Chapter 4, it would be best to use the UCST polymer to produce fibre structures and explore their thermo-responsive performance. For LDHs nanosheets in Chapter 6, other less toxic paramagnetic metal ions, such as Mn, rather than Gd, can be explored to produce theranostic systems. For the PCL/PLGA-based implantable systems prepared in Chapter 7, the drug loading amount and release profile can be further optimized, so as to provide implantable systems giving drug release over different periods and supplying various drug amounts. This can be achieved by varying the polymer-drug-LDH ratio, changing the core-shell ratios, and optimizing the electrospinning process.
8.2.3. \textit{In vivo} Studies

In the longer term, the next stage is to explore the theranostic formulations using \textit{in vivo} models. These will involve test the anti-cancer therapeutic efficacy using murine tumour xenograft models. Specifically, UCST microparticles produced in \textit{Chapter 5} and Gd-based layered double hydroxide nanosheets in \textit{Chapter 6} can be tested using a solid tumour via intramuscular injection. Core-shell fibre fabricated in \textit{Chapter 7} can be implanted into glioma rat models. The dosage of formulation applied can be optimized to obtain a desirable anti-cancer effect. The tumour size and corresponding biomarkers affected by chemotherapeutic drugs should be continuously evaluated during the \textit{in vivo} studies, and also at the end of the experiments. MRI images can also be obtained periodically, to test the practicability to monitor the drug release based on MRI signal intensity.
Appendix 1: Morphological Characterization Data of Fibres@Gd(DTPA) or SPIONs/PDA

Figure A1-1 SEM images and Mg, Al and N elemental mapping of G-P-PLGA1.

Figure A1-2 SEM images and Mg, Al and N elemental mapping of G-P-PLGA2.
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Figure A1-3 SEM images and Mg, Al and N elemental mapping of S-P-PLGA1.

Figure A1-4 SEM images and Mg, Al and N elemental mapping of S-P-PLGA2.
Appendix 2: XPS Spectra of Fibres@Gd(DTPA) of SPIONs/PDA

Figure A2-1 Survey scan XPS spectra (A, B), and the C 1s (C, D), and O 1s (E, F) regions of PCL-LDH@PLGA1 and PCL-LDH@PLGA2.
Figure A2-2 XPS spectra of P-PLGA1.

Figure A2-3 XPS spectra of P-PLGA2.
Figure A2-4 XPS spectra of S-P-PLGA1.

Figure A2-5 XPS spectra of S-P-PLGA2.
Figure A2-6 XPS spectra of G-P-PLGA1.

Figure A2-7 XPS spectra of G-P-PLGA2.
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Figure A2-8 Survey scan XPS spectra of (A) PCL-LDH@PLGA1 and (B) PCL-LDH@PLGA2 after dissolution for 432 h.

Figure A2-9 Survey scans XPS spectra of the post-treated fibres (as labelled) after dissolution for 432 h.
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Appendix 3: Viability Data for PCL-LDH@PLGA1/2 based materials

Figure A3-1 Viability data for monolithic PLGA1 and PLGA2 fibres.

Figure A3-2 Viability data for PCL-LDH@PLGA1/2 based materials (A, Caco-2; B, A549).