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**CAVITATION-ENHANCED TUMOUR-TARGETING
VIROTHERAPY BY ULTRASOUND**

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Abstract

Systemic administration of adenovirus type 5 (Ad5) vectors for the treatment of cancer is limited by poor circulation kinetics and inefficient uptake from the bloodstream into tumours. This study reports a novel method for linkage of highly-PEGylated gold nanoparticles (AuPEG) to Ad5 by a single reduction cleavable bond. The resulting ‘dandelion’ structure provides very effective steric shielding with only minimal and reversible modification of the Ad5 capsid. This ablates *in vitro* cell infection, improves protection against the binding of antibodies, and enhances *in vivo* circulation kinetics.

Focussed ultrasound is a promising technology for the non-invasive, targeted treatment of cancer. In the context of drug delivery, cavitation energy generated upon exposure of ultrasound contrast agents to focussed ultrasound can be used as a powerful stimulus to move therapeutics over distances of hundreds of microns away from blood vessels. In addition to providing a platform for effective stealthing, conjugation of AuPEG to Ad5 also increases the effective density of Ad5. This increase in density imparts a second major advantage on the strategy, observed for the first time in the present study: denser particles are transported significantly farther by cavitation-induced microstreaming than identically-sized particles of lower density. Specifically, in *in vitro* tests using a tumour-mimicking flow-channel phantom model and in *in vivo* experiments using tumour bearing mice, Ad5–AuPEG was delivered farther from vessels in response to ultrasound induced cavitation than either naked Ad5 or polymer-coated Ad5.

The enhancements in stealthing and improvements in response to ultrasound provided by this strategy enabled up to 12% (S.D. 0.97) of the injected dose to be deposited in the tumour, compared to just 0.12% (S.D. 0.05) for Ad5 without ultrasound ($p < 0.001$). Consequently, in a survival study, mice treated with Ad5–AuPEG with focussed ultrasound had the slowest tumour growth and longest survival rate when compared to mice treated with Ad5 alone, Ad5–AuPEG alone, or Ad5 with focussed ultrasound. These results provide compelling evidence that the combination of focussed ultrasound with density-augmented stealthed Ad5 results in improved delivery to tumours and therapeutic efficacy.

This combination of ultrasound with particle modification for optimal cavitation-mediated delivery has the potential to be applied to a broad range of anti-cancer nanomedicines and therapeutics to augment their bio-availability for improved cancer treatment.

Statement of Originality

I hereby declare that this submission is my own work and, to the best of my knowledge, it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at the University of Oxford or any other educational institution, except where due acknowledgement is made in the thesis.

Any contribution made to the research by others, with whom I have worked at the University of Oxford or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

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List of abbreviation

Ad5	Ad5enovirus serotype-five expressing GFP under CMV control (non-replicating; Δ E1/E3); used in all <i>in vitro</i> and most <i>in vivo</i> (except the survival study) experiments in this DPhil study.
Ad5-mir122	Wild-type Ad5 containing four mir122 binding sites in the E1A 3' untranslated region (replicating)
Ad5-mir122-luc	Wild-type Ad5 containing four mir122 binding sites in the E1A fused to luciferase (replicating)
ANOVA	Analysis of variances
AU	Absorbance unit
AUCF	Amicon ultra-4 centrifugal filter
Au	Gold nanoparticles
AuPEG	PEGylated gold nanoparticles
BME	β -mercaptoethanol
CAR	Coxsackie and adenovirus receptor
cm	Centimetre
CMV	Human cytomegalovirus
CsCl	Caesium chloride
ddH ₂ O	Doubled distilled water
DMEM	Dulbecco's modified Eagle's Media
DNA	Deoxyribonucleic acid
DLS	Dynamic light scattering
ELISA	Enzyme-linked immune sorbent assay
f_0	Centre frequency of a transducer
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FFT	Fast Fourier Transform
FITC	Fluorescein isothiocyanate
FX	Factor X
g	Gram
g	Gravitational force ($6.674 \times 10^{-11} \text{ N m}^2 \text{ kg}^{-2}$)
GFP	Green fluorescent protein
HIFU	High intensity focussed ultrasound
HINGS	Heat inactivated goat serum
hr	Hour(s)
Hz	Hertz
i.v.	Intravenous(ly)
KC	Kupffer cells
kb	Kilobases; 1,000 units of DNA
kg	Kilogram
kHz	Kilohertz
kPa	Kilopascal
L	Litre
M	Molar or molarity (moles/L)
MFI	Mean fluorescence intensity

MHz	Megahertz
min	Minute(s)
mg	Milligram
mL	Millilitre
mM	Millimolar
mm	Millimetre
MMB	Magnetic microbubbles
MMP	Matrix metalloproteinases
MPa	Megapascal
mV	Millivolt
MW	Molecular weight
μL	Microlitre
μM	Micromolar
μm	Micrometre
nm	Nanometre
nM	Nanomolar
NTLS	Non-thermosensitive liposomes
NUS	No ultrasound
PCD	Passive cavitation detector
PBS	Phosphate-buffered saline (pH 7.2 unless otherwise indicated)
PEG	Polyethylene glycol(s)
PHPMA	Poly-[N-(2-hydroxypropyl)methacrylamide]
PRF	Pulse repetition frequency
PRFP	Peak rarefactional focal pressures
QA	Quaternary amine
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
sec	Second(s)
SEM	Scanning electron microscopy
siRNA	Small interfering RNA
SS	Disulphide bond
TEM	Transmission electron microscopy
TGA	Thermal gravimetric analysis
TLS	Thermosensitive liposomes
TNBS	Trinitrobenzenesulfonic acid
UCA	Ultrasound contrast agent
US	Ultrasound
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
v.p.	Virus particles
W	Watt

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Physiology of cancer

In developing novel strategies for cavitation-enhanced tumour-targeted virotherapy, understanding of the physiologic environment of cancer cells and malignant tumours is crucial. The following three sub-sections (tumour formation, angiogenesis and vasculature structure, and nanoparticles as cancer therapy) provide an overview of the current cancer knowledge and research.

1.1.1 Tumour formation

Cancer usually arises from the mutations in genes that regulate cell division. While some mutations are inherited, most are induced by external factors such as chemicals, UV light, X-rays, tobacco products etc. [1]. Usually, at least five to ten such mutations are needed for a normal cell to evolve into a cancer cell [1, 2]. When enough mutated genes have accumulated within cells, they will divide irregularly and faster than normal healthy cells, eventually leading to the growth of a tumour mass [3] [2].

There are two types of genes associated with cancer: oncogenes and tumour suppressors. While oncogenes make cellular proteins that enhance cell division or inhibit apoptosis (the process of programmed cell death), tumour suppressors produce proteins that prevent cell division or result in apoptosis [4]. As a result, mutations in oncogenes accelerate cell growth, yet mutations in tumour suppressors prevent the normal apoptosis happening; in either case, uncontrolled cell division occurs [2]. Oncogene is 'gain of function' mutation because when the gene mutates, the newly created protein gains a function to enhance cell division), while tumour suppressor is 'loss of function' mutation because when the gene mutates, the new protein loss its apoptosis function. Except sperm and egg cells, all human cells have two copies (alleles) of each gene; oncogene is a dominant gene because only one

such copy is needed to mutate to cause cancer, yet tumour suppressor is a recessive gene because both copies are required to mutate to cause cancer [1].

There are four major phases of tumour formation: mutation, carcinoma *in situ*, invasive stage, and metastasis. A common misconception is that ‘tumour’ and ‘cancer’ are synonymous. However, a tumour can be benign, pre-malignant, or malignant, whereas cancer is by definition always malignant [5]. The four phases of tumour formation should not be confused with the four stages of cancer, which will be explained later in this sub-section.

The first phase occurs when some oncogenes or tumour suppressors in regular cells mutate to lose their apoptotic function [3]. This process may take place over many cell divisions. The number of progeny cells produced becomes much higher than the number of cells currently undergoing apoptosis, leading to the formation of an abnormal mass of tissue known as neoplasm or as the primary tumour [4].

The second phase is called carcinoma *in situ*, which is defined by the presence of cancerous cells that have not yet invaded other tissues; in other words, the neoplastic cells proliferate in their original tissue [6]. Usually this phase lasts between five and ten years in humans, and is limited by the efficiency of and amount of nutrition flow to the neoplasm. By triggering the induction and growth of new blood vessels (angiogenesis) towards itself a neoplasm can overcome this limitation and thereby ensure the provision of the nutrients required for growth and spread. If this is achieved the primary tumour will proceed to the next phase called the invasive stage [2].

The invasive phase, the third phase, can occur promptly (e.g. only a few months) right after carcinoma *in situ*, but it may also take up to five years in some cases [7]. This particular phase is heavily dependent on the ability of the neoplasm to grow towards the neovasculature, to mediate more irregular blood vessel formation inside the tumour or adjacent tissue, and to trigger degradation of basement membrane structures [2, 8]. During this phase, matrix

metalloproteinases (MMPs) are highly expressed in most cancer cells and capable of degrading extracellular matrix to assist the development from invasive phase to metastasis. In addition, MMPs can alter cell-cell interactions and enhance angiogenesis, a crucial step to sustain tumour growth [9, 10].

The final phase, metastasis, indicates the spread of cancer cells into other organs of the body [2, 11]. Cancer cells can break away from a primary tumour and enter the blood stream or lymphatic system (part of the immune system that produces, stores, and carries cells that fight infections) [11]. The duration required for the development of metastasis can range from a few months to five years [2]. Studies have shown that MMPs are essential in creating and maintaining an environment to support the development of metastatic tumours; as a result, the development of MMP inhibitor drugs have been going on a prominent research field this past decade [9, 10].

It is also essential to differentiate the four phases of tumour formation from the four stages of cancer [12, 13]. While the former has been discussed above, the latter is a description of the extent cancer has spread and can be divided into five stages [13, 14]. Stage 0 refers to the initial development of carcinoma *in situ*. Stage I indicates the cancer is moderately small and is confined to its organ of origin. Stage II implies that the cancer is significantly larger than that of stage I but has not yet spread to surrounding tissues. Stage III designates that cancer has spread to surrounding tissue and, in some cases, lymph nodes. Stage IV, the last stage, occurs when cancer has metastasised to other distant organs of the body. Stage IV cancer is sometimes called secondary or metastatic cancer [12, 14].

Usually stage 0 patients might have some elevated expression of cancer proteases, but unless one performs a very sensitive analysis of bloodstream composition on regular basis, cancer at this stage is rarely detectable [15]. Stages I tumour can be surgically removed if deemed small enough. Patients with stages II to IV cancer are typically treated with

chemotherapy, radiotherapy, surgery, or a combination of these therapies [16, 17]. The five-year survival rate of cancer patients treated with these therapies at various stages was reported to be 93% in Stage I, 86% in Stage II, 69% in Stage III, and 50% in Stage IV [18].

1.1.2 Tumour angiogenesis and vasculature structure

In order to develop effective methods for cancer therapy, the mechanisms of angiogenesis (the development of new blood vessels) within the tumour and the structure of the vasculature surrounding tumours must first be understood. Angiogenesis is a vital step in the transition of tumours from a benign state to a malignant one [2, 8]. Without the support of blood vessels, a tumour usually cannot grow to more than 1-2 mm³ in size because it lacks the supply of oxygen and other essential nutrients which cannot effectively diffuse over distances greater than 180-200 µm [19].

As a tumour grows larger in size, it will eventually induce the growth of blood vessels in its surroundings by secreting various growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [8]. These growth factors can induce the growth of capillary vessels in the tumour, enabling the tumour to expand by providing a supply of oxygen and other nutrients to meet its high metabolic requirements [11]. Besides providing the necessary nutrients, angiogenesis also serves as a waste pathway by allowing the biological end products secreted by actively dividing cancer cells to escape into the main circulatory system of the body [20].

Metastasis, the spread of a tumour to other organ systems of the body also requires angiogenesis. Individual cancer cells can break free from a well-established primary tumour, move into the newly formed blood vessels, and be transported to a different organ or tissue, where they become embedded and initiate the growth of a secondary tumour [2, 12].

The majority of cells in the human body are only a few cell diameters away from a blood vessel, making the delivery of oxygen and nutrients to the cells very efficient. However, in tumours, because cancer cells proliferate at a much faster rate than normal cells, there is a much lower ratio of blood vessels per unit volume of cells compared to that of normal tissue [21]. The rapid expansion of cancer cells inside a tumour pushes under-developed capillaries apart, meaning a significant portion of the tumour cells are distant from blood vessels and resulting in inadequate draining of interstitial fluid [22]. Together, these circumstances create a disordered vascular structure [23]. Normal vessels (**Figure 1-1a**) are well organized and have consistent diameters, whereas those from a cancer are dilated, irregular, and tortuous (**Figure 1-1b**).

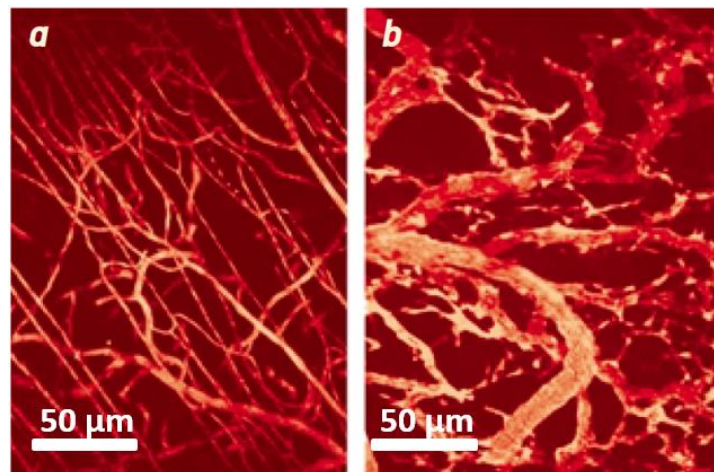


Figure 1-1: Images taken of normal and cancer vasculatures. Normal vessels (a) are well organized and have even diameters, whereas those from a colon cancer (b) are dilated and tortuous. Images were taken using intravital microscopy directly from human patients; reproduced from [23] with permission of *Scientific American*.

While in normal vasculature, there is a strong correlation between vessel diameter and red blood cell velocity (**Figure 1-2**, left), in tumour vasculature, there is a low correlation and also much average lower flow rate (**Figure 1-2**, right). Blood flow rate in normal vasculature is directly proportional to the pressure gradient between arterioles and venules, but blood flow in tumour vasculature shows a reduced pressure gradient [24]. While blood flow in normal vasculature is always in a single direction, observations have been made such that blood flow

may accelerate, decelerate, or even reverse in direction within the same tumour vessel [22]. Furthermore, geometric resistance offered by tumour vessels is higher due to their peculiar geometry and branching patterns [22]. This poor vascular architecture, along with the lack of functional lymphatic drainage, does not allow efficient movement of metabolic waste products (e.g. lactic acid) out of the tumour, lowering the surrounding extracellular pH [25].

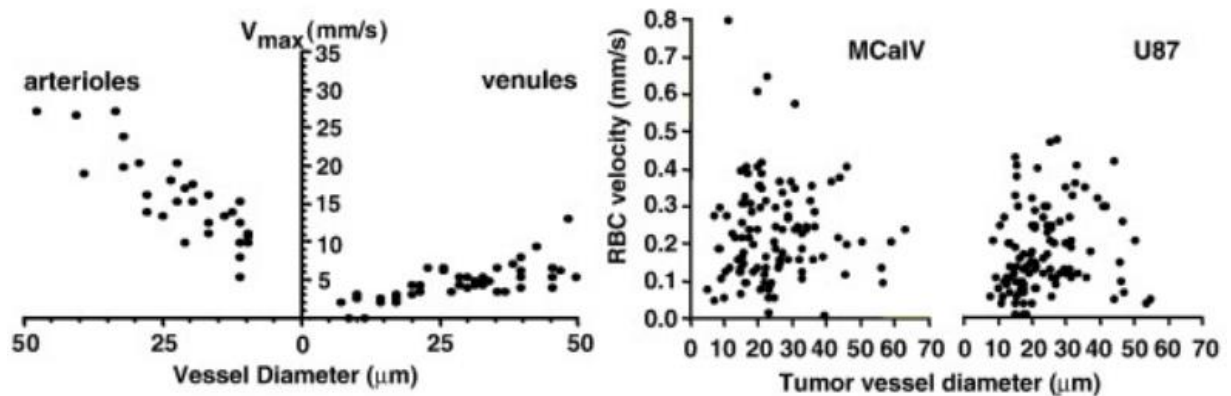


Figure 1-2: Comparison of blood flow rate in healthy and tumour vessels. Blood flow determined by intravital microscopy in normal vessels (left graph) and tumour vessels in MCalV and U87 tumours (right graph). 150kDa fluorescence-labelled dextran molecules were intravenously injected into healthy rabbits and mice implanted with MCalV and U87. Intravital microscopy was used to determine the velocities of the dextran beads at rabbit ear chamber for normal vessels and mouse dorsal tumour skin chambers tumour vessels. Each point above represents one sample; reproduced from [24] with permission of *Microvascular Research*.

One key component of cancer progression is the leakiness of the blood vessels supplying tumours; by understanding the structural basis of the tumour leakiness, the penetration of drugs and other cancer therapy agents into tumours can be understood. From a MCal-IV tumour-cell study which analysed over 300 tumour vessels from 6 individual tumours (**Figure 1-3**), the median blood vessel diameter was found to be 25 μm while the mean blood vessel diameter was 39 μm [26]. Once drugs or any other cancer therapy agents enter the blood vessels, the next target is the gaps between neighbouring tumour associated endothelial cells which have been calculated to be between 0.4 and 0.6 μm [27] or 0.1 and 1 μm [28]. After passing through the endothelial gaps, these therapeutic agents need to pass through intercellular spaces before exposure to tumour cells can be achieved. The same study

(Figure 1-4) examined the size distribution of hundreds of intercellular spaces within MCa-IV tumours—with a mean and medium length values of $1.7\ \mu\text{m}$ and $1.5\ \mu\text{m}$, respectively [26].

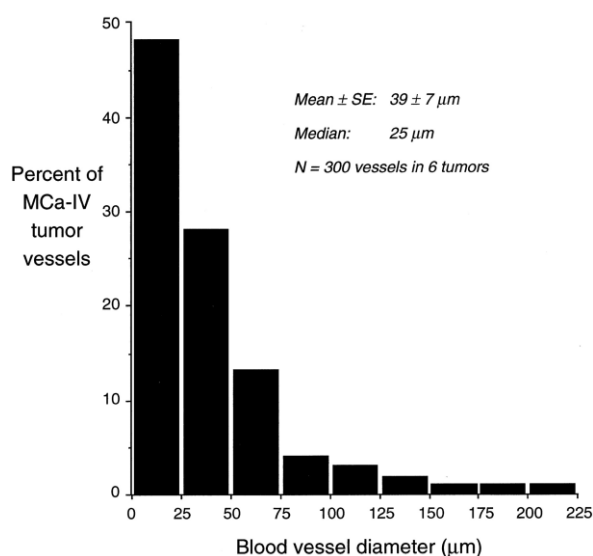


Figure 1-3: Size distribution of vessels in MCa-IV tumours. Diameters of 50 blood vessel profiles were measured in cross sections of each of 6 tumours. Tumour vasculatures were visualised by cutting off MCa-IV tumour cross sections, reacted with fluorescence-labelled lectin (1 mg/mL), fixed with 0.5% glutaraldehyde and 1% paraformaldehyde in PBS, and imaged under fluorescence microscope; reproduced from [26] with permission of *Cancer Research*.

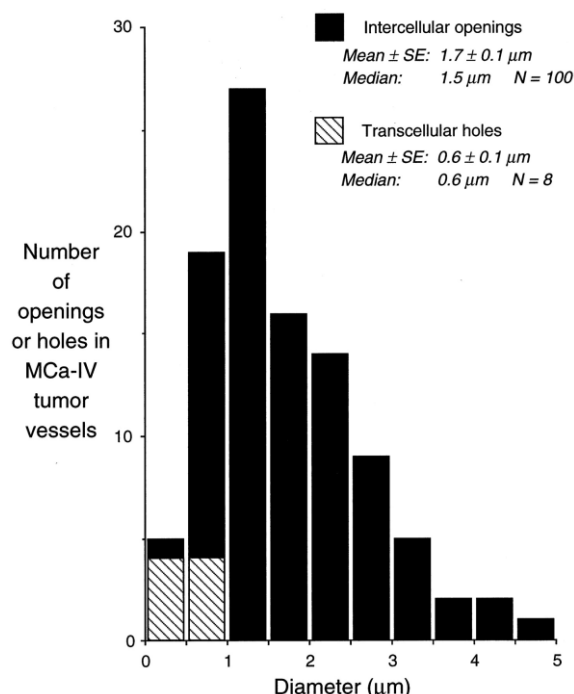


Figure 1-4: Size distribution of 100 intercellular spaces and 8 transcellular holes, found in a sample of 700 endothelial lining cells from blood vessels of MCa-IV tumours. Tumour intercellular spaces were visualised from the same histology staining technique was used as describe in Figure 1-3 above; reproduced from [26] with permission of *Cancer Research*.

The leaky vasculature and extensive deregulated angiogenesis within tumours can greatly enhance the movement of macro-molecules and anti-cancer drugs through the leaky vasculature and into the tumour interstitium — a phenomenon known as the enhanced permeability and retention (EPR) effect. The increased vascular permeability also provides adequate supply of oxygen and cellular nutrients for rapid tumour growth and development in the perivascular region [29]. The EPR effect becomes vitally important for giving longer circulating drugs or therapeutics the ability to passively accumulate at a higher concentration at tumour sites than within normal tissues [30]. Heneweer and colleagues used zirconium-labelled albumin to test the how EPR effect enhance its accumulation in three xenograft models of human cancers (CWR22rv1, DU-145, and PC-3) on mice, and concluded that the 1-hr albumin accumulation in muscle increased from 0.6% to 2.7% for tumour implanted on murine muscle [31]. However, the flip side of this unregulated influx of macromolecules and fluid into the tumour through leaky vasculature is that the interstitial pressure increases dramatically, creating a barrier for movement of molecules beyond the perivascular space [21]. EPR effect is considered a type of passive targeting; on the other hands, to achieve active targeting, the surfaces of anti-cancer therapeutics (e.g. liposome, polymers, and therapeutic nanoparticles) are conjugated to cancer-binding molecules (e.g. ligands) that can bind to specific target tumour cell receptors. The contact between active targeting therapeutics and the infected cancer cells will lead to internalisation or release of the entrapped drugs. While active targeting can be very specific and reduce the side effects of drugs, it is more complicated to be synthesised and decreased the ability to adjust drug dosage.

In summary, tumour blood flow, and the EPR effect are important to tumour growth, detection, and treatment. Tumour blood flow is spatially and temporally heterogeneous, and depends on the host-tumour interaction. Blood flow is proportional to arterial or venous pressure difference and inversely related to geometric and viscous resistances. Both geometric and viscous resistances in tumours are higher compared to several normal tissues. The

metabolic micro-environment of tumours and down-regulated perfusion results in acidosis and hypoxia in certain tumour regions that can induce drug resistance (**Figure 1-5**) [32].

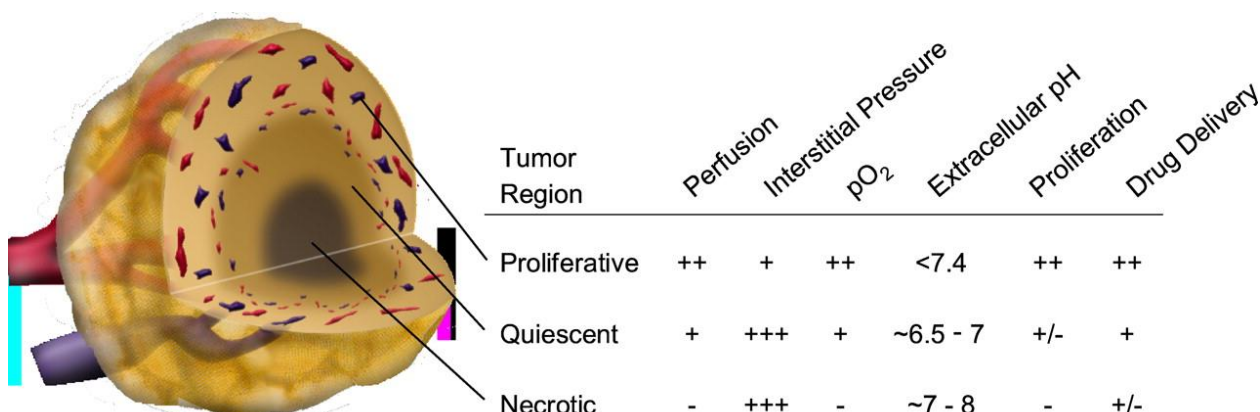


Figure 1-5: Schematic diagram of three micro environmental regions in a centrally necrotic tumour. A spontaneous tumour may consist of many such necrotic foci. Decreasing magnitude of various physiological parameters is indicated as +++, ++, +, +/-, and -; reproduced from [32] with permission of *Proceedings of the National Academy of Sciences*.

Knowledge of the tumour vessel blood flow rate, the disorganised pattern of tumour vessels, the relatively lower pH of the tumour micro-environment, the size of the tumour vessels, and the size of tumour intercellular spaces help to enable the design of therapeutic agents and strategies to enable successful delivery to, and throughout, tumours. Hence, the last section of this chapter will now discuss the current state of the art in cancer therapy.

1.1.3 Cancer therapy

Primary surgery can only efficiently remove defined tumour from stage I patient [33]. However, for patients with cancer stages II to IV, a wide array of clinical and experimental cancer therapies have been developed over the past decades, including low molecular weight chemotherapies, gene therapies, virotherapies, anti-cancer vaccines, photodynamic therapies, radiation therapies, physical therapies such as focussed ultrasound, biological targeted therapies, and therapeutic nanoparticles [34-36].

Gene therapy is the insertion, alteration, or removal of genes within target cancer cells, transforming their cellular activities to behave more like normal cells (e.g. to trigger apoptosis) or to express cytotoxic proteins [34, 35]. Anti-cancer vaccines are usually vaccines

that treat existing cancers, prevent infection by cancer-induced viruses, or prevent further progression of cancer in individuals [35, 37]. Photodynamic therapy is a cancer treatment that uses a photosensitizer, which, under the exposure to a specific wavelength of light, will produce oxygen radicals that kill nearby cancer cells. Photodynamic therapy is performed to eradicate early-stage tumours and to reduce their size, so the tumour will not develop into a malignant cancerous tumour [36, 38]. Radiation therapy applies ionizing radiation to control the growth of malignant tumour cells [35, 39]. Therapeutic ultrasound uses alternating compressional and rarefactional pressure perturbations (or waves) occurring at a frequency between 0.5 and 5 MHz to damage tumour tissues or modulate the tumour microenvironment by either mechanical or thermal mechanisms [36, 40]. Biological targeted therapy is a strategy that uses small molecules and monoclonal antibodies to interact with specific target molecules needed for tumour growth and survival, thus blocking the growth of cancer cells [35, 41]. Therapeutic nanoparticles appear to be good candidates to improve the bio-distribution of cancer drugs because their surface charge and size (usually < 100 nm) can be tuned to improve the circulation time in bloodstream and EPR assisted passive accumulation of drug cargo in tumours [36, 42].

Chemotherapy refers to the killing of cancer cells using cytotoxic drugs [43]. Chemotherapy is usually used with either surgery or radiation therapy. Since chemotherapeutic drugs target and kill cells that divide rapidly, it will kill both cancer cells but also healthy cells that undergoes fast growth ate such as blood cells produced in bone marrow and digestive tract [44]. In addition, patients treated with chemotherapy easily develop resistance to platinum-based chemotherapy, making later treatment very difficult [43]. Virotherapy is the delivery of replicative cell-specific viruses to tumours [45, 46]; however, virotherapy has always been given in conjunction with chemotherapy in clinical trials to reduce the overall drug-resistance rate [47]. The major reason for using virotherapy is

that it uses very different and multiple mechanisms (explore in more depth in section 1.2) to kill cells and therefore the tumour is less likely to become resistant [46, 47].

Most cancer research groups specialise and focus on one of these cancer therapies, but a few laboratories have attempted to combine two of these therapies. For example, chemotherapy has been combined with therapeutic nanoparticles [48], gene therapy with radiation therapy [49], anti-cancer vaccine with photodynamic therapy [50], virotherapy with therapeutic ultrasound [45], and therapeutic nanoparticles with therapeutic ultrasound [51]. These combination therapies aim to enhance the efficacy of cancer therapy.

The main objective of this DPhil project is the integration of three experimental cancer therapies (virotherapy, therapeutic ultrasound, and therapeutic nanoparticles) into one novel combination therapy, which combines the advantages of the three existing therapies. Based on the latest literature review (as of September 2013) of these fields, this proposed combination therapy has never been attempted previously, and an extended literature review of these cancer therapies forms the remaining section of this chapter. Section 1.2 discusses the current development and limitations of virotherapy. Section 1.3 gives a brief overview of therapeutic ultrasound and the physical mechanisms by which therapeutic ultrasound enhances drug delivery to tumours. Section 1.4 reviews current applications of therapeutic ultrasound for nano-medicine and therapeutic nanoparticles. Section 1.5 discusses the safety of virotherapy and therapeutic ultrasound. Lastly, section 1.6 summarises all reviews and suggests key implications of current work on ultrasound-enhanced virotherapy.

1.2 Oncolytic adenovirus

Virotherapy can be classified as a type of gene therapy where by viral particles (v.p.) can naturally, or can be genetically engineered to, selectively infect and kill cancerous cells whilst leaving healthy cells relatively unaffected. Even though both virotherapy and gene therapy refer to the delivery of specific anti-cancer genes to targeted cancer cells, virotherapy

provides the capacity for virus replication inside host tumour cells, and due to the lytic life cycle of the virus the eventual death of the host cells. For a typical example, such as wild type adenovirus, this can provide a 10,000-fold amplification of the delivered dose with 72 hr of exposure, and as the progeny viruses are infective they can then potentially continue to propagate through the tumour. No other therapy class allows the therapeutic agent to be amplified in this way within the target cells [52, 53]. The most commonly used virotherapy vehicles are adenoviruses, reoviruses, and herpes simplex viruses, and they have been tested for utility in treating a range of cancers [47]. While section 1.5.1 will address these various forms of oncolytics and their use as adjuncts in clinical trials, the remaining literature review focuses on oncolytic adenovirus, which is most relevant to the present work.

Isolated in 1953 by Rowe *et al.*, adenovirus was first identified as an agent that caused upper respiratory tract infection in humans [54]. Since its isolation, Ad5 has been used to investigate several important molecular biology processes including mRNA splicing, cell cycle control, immune response regulation, and cancer therapy [55]. To date, 57 serotypes of human adenovirus have been identified, which are classified based on their ability to resist neutralisation by antibodies encountered against other types of Ad5 [56]. These serotypes are categorised into one of the seven groups, denoted ‘A’ to ‘G’, based on characteristics such as DNA homology, oncogenic potential, and agglutination of red blood cells [57]. Out of all 57 adenovirus serotypes, adenovirus serotype-5 (Ad5) in group C has been the most commonly chosen viral vector, due to its highly efficient, regulated and well understood pathway of infection, and its track record of clinical safety [58]. Ad5 therefore served as the only adenovirus used in this study, and is extensively reviewed for the remaining of section 1.2.

As shown in **Figure 1-6**, Ad5 consists of a 36-kb double-stranded linear DNA genome encased within an icosahedral particle (20 triangular facets) composed of 12 distinct proteins. The core capsid of Ad5 is around 60 to 90 nm, and the trimeric fibre of each Ad5 extends around 30 nm outward from the capsid, making the average total size of Ad5 between 90 and

120 nm. It should be noted that fibre length depends on adenovirus serotype (e.g. groups B and D have much shorter shaft length than group C, which includes Ad5). Each fibre monomer contains three regions: a ‘knob’ domain (responsible for binding to coxsackie and adenovirus receptor, CAR), a long ‘shaft’ (for structural support), and a ‘tail’ (responsible for attaching to other capsid proteins) [59]. Each Ad5 fibre consists of three identical intertwined strands with the KKTK motif, which was thought to be an essential binding site for heparin sulphate proteoglycan (HSPG) membrane receptor in 1999 [60]. However, in 2006, Bayo-Puxan and colleagues mutated the KKTK motif of Ad5 and found that this motif has no role in HSPG interactions [61], and it is now widely accepted that this motif is essential to allow the bending of the fibre shaft which then lead to the binding of Ad5 to CAR and integrins [61, 62]. The homotrimer fibre non-covalently complexes/attaches to the penton base protein [59], and this might suggest why most fibres were often detached from the Ad5 capsid under transmission electron microscopy (TEM) imaging since the acceleration of the electron beam can easily break these non-covalent linkage apart [63].

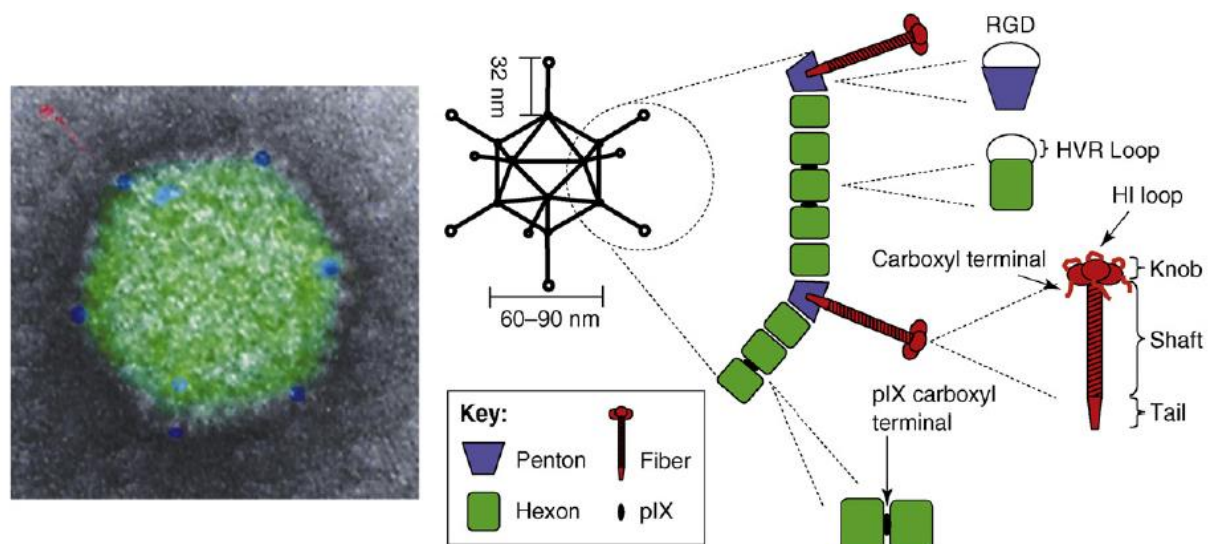


Figure 1-6: Representation of the adenovirus serotype-5 (Ad5) structure. (Left) TEM image of Ad5, where the capsid hexon proteins were stained green and the pentose proteins were stained blue; on the top left of the image, there is a detached fibre which was stained red. (Right) Each Ad5 possesses an icosahedral morphology with a core diameter of 60–90 nm and a flexible fibre extending 32 nm from each of the 12 penton-base vertices. The viral capsid consists of three major proteins: hexon, penton, and fibre, in addition to several proteins, such as pIX, that stabilise hexon and penton; reproduced from [59] with permission of *Trends in Biotechnology*.

In addition to the fibre (polypeptide IV; 62 kDa), the major capsid proteins of Ad5 are the hexon protein (polypeptide II; 130 kDa) and the penton (polypeptide III; 82 kDa). Each viral particle (v.p.) contains 720 hexon monomers, which in addition to their structural role have also recently been demonstrated to provide factor X mediated binding to cells [64, 65]. The 20 triangular facets are joined by 12 vertices which are the exact location of the penton-base complexes, where polypeptide loops containing Arg-Gly-Asp (RGD) amino acid sequence are responsible for Ad5 binding to secondary cell integrin receptors to trigger the endocytotic uptake of v.p. by the infected host cells [66]. In addition, macrophages produced from spleen mediated inflammatory responses possess such integrin, making these white blood cells easily bind to penton region of Ad5 for endocytosis [66]. The 14-kDa Protein IX (pIX) is present to stabilise the overall structure of hexons and pentons [59]. In addition, minor proteins VI and VIII are inside the capsid and directly support the internal structure of hexons. Proteins V and VII are core proteins that stabilise internal viral genome [67].

The infection (entry) of Ad5 into host cell is a two-step process: a docking step followed by endocytosis (**Figure 1-7**). The docking phase begins when the fibre knob binds to the target cells via a membrane coxsackie and adenovirus receptor (CAR) [68], and this triggers the interaction of the RGD motif in the penton base with $\alpha v\beta 3/\alpha v\beta 5$ integrins, which consequently activates endocytosis of the Ad5 [69]. The penton-integrin interaction results in integrin clustering which activates several signal kinase pathways to reorganise actin filaments, which reorganise plasma membrane forming an Ad5 engulfing endosome [70]. This viral internalisation is mediated via clathrin-associated endocytosis [71]. The acidification of the endosome due to the activity of cellular proton pumps disassembles the capsid, releasing the membrane lytic protein VI which modulates the endosomal escape to release the nucleo-capsid [72]. Cytoplasmic dynein facilitates the attachment of the nucleo-capsid to the microtubule network that further assists the final movement to host cell's nucleus [73, 74]. At the site of nuclear pore, the nucleo-capsid interacts with CAN/Nup214

and binds with Hsc70 to recruit histone H1 and import factors (importin β and importin 7), which eventually leads to the total disassembly of the nucleocapsid and delivery of viral DNA to the nucleus [75].

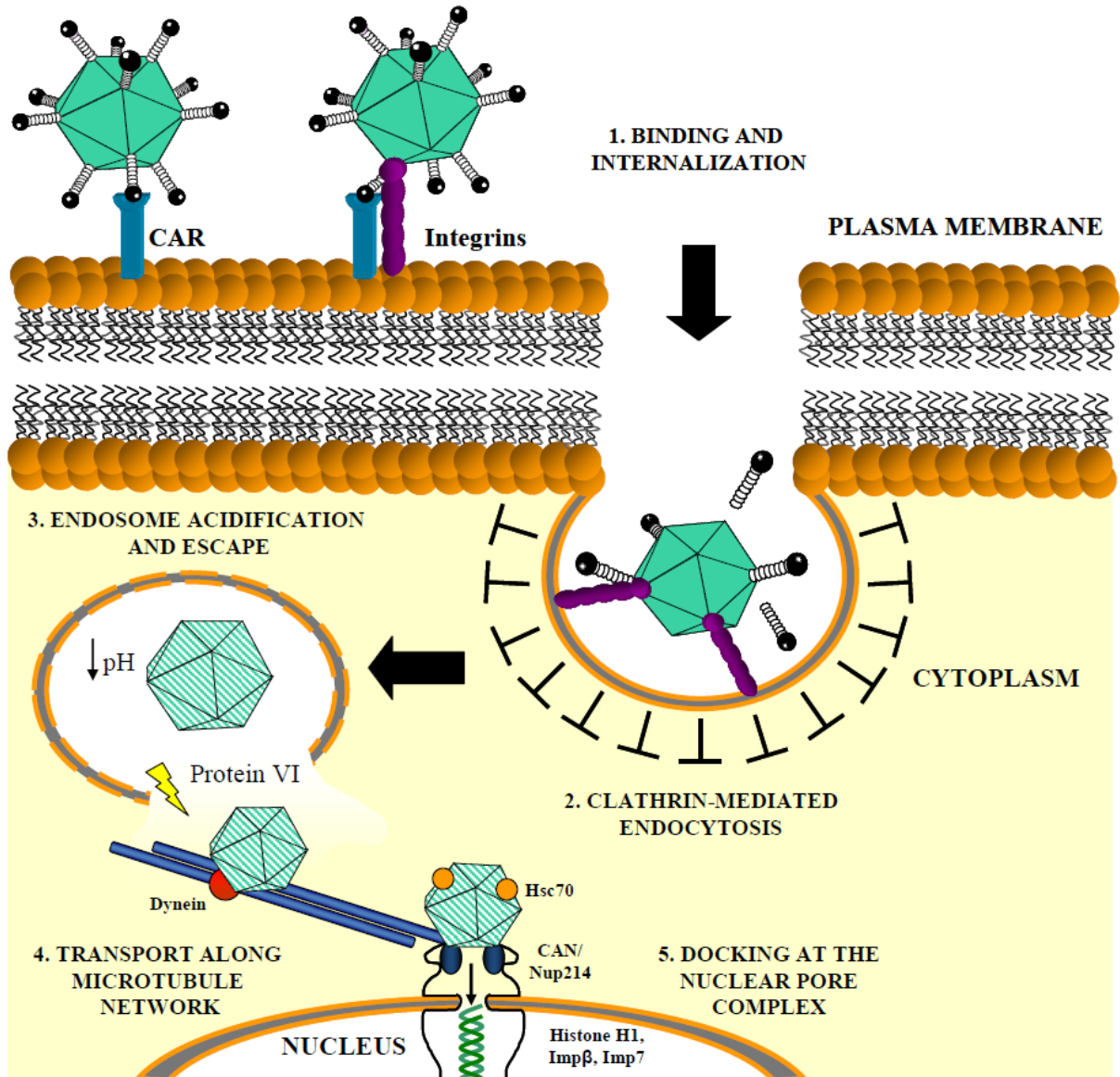


Figure 1-7: Entry pathway of adenovirus (Ad5) and the delivery of viral DNA to host's nucleus. The entry pathway of Ad5 requires a docking phase and endocytosis, followed by endosomal escape; reproduced from [67] with permission of *Viruses*. A detailed description of the procedures can be found in the following paragraph.

Although Ad5 binds to CAR for the docking phase, other adenovirus groups may bind to different surface receptors to initiate similar docking phase; for examples, adenovirus types 3 and 7 bind to cluster of differentiation-46 [76], adenovirus types 11 and 14 bind to

desmoglein-2 [77], and adenovirus type 37 binds to glycoprotein-D1a [78]. In other words, the binding of any adenovirus serotype to its host's surface receptor is very specific.

The 36-kb Ad5 genome consists of two major non-contiguous overlapping regions: early and late (which is defined based on the time of transcription after infection). Within the first 6 hr after infection, the early genes (E1A, E1B, E2A, E2B, E3, and E4) which are involved in regulation of viral replication, are transcribed and translated. The late genes (L1 to L5) encode viral structural proteins (hexon, penton, fibre) and are usually produced more than 7 hr after the infection [79]. E1A is arguably the most influential early gene which inhibits pathways of programmed cell death, activates transcriptions of other early genes, and amplifies viral gene expression [80]. As a result, the deletion of E1 results in a non-replicative virus. In addition, studies have also shown that the immune modulatory protein E3 is not crucial for Ad5 replication in vitro in cultured human cells. Hence, commercially available, safe, non-replicative Ad5 usually has E1 and E3 genes deleted [81]. HEK 293 cells have been the most popular choice to clone both replicative and non-replicative Ad5 since they have been engineered to contain a 4.5 kb Ad5 DNA fragment encoding both E1A and E1B within chromosome 19, which helps to amplify replication of any Ad5 whether the E1 gene is deleted or not [82]. Besides the survival study (Chapter 5) conducted in the last part of these studies which required replicative Ad5, all other experiments with Ad5 carried out here used non-replicative Ad5 Δ E1/E3.

1.2.1 Current limitations

Oncolytic Adenovirus type 5 (Ad5) vectors have been engineered to infect cancer cells selectively, self-amplify and then achieve lytic release and infection of neighbouring cancer cells [83, 84]. This selectivity and power makes Ad5 one of the most promising nano-medicines under development, and Ad5 vectors have shown good anti-tumour efficacy when delivered by intra-tumoural injection [85]. However, several limitations have prevented the

intravenous (i.v.) delivery of Ad5 for the treatment of metastatic cancer, i.e. the disease in its most common and fatal form [67, 86]. Notably, rapid blood cell and reticuloendothelial system mediated clearance, poor target cell selectivity, and instantaneous antibody binding lead to efficient sequestration and neutralisation of i.v. delivered Ad5 before it can reach target tumours [87]. To increase the potency of systemically administered Ad5, its circulation half-life must be increased and binding to non-target cells avoided [88]. Several Ad5 capsid proteins are responsible for both its cellular tropism and *in vivo* sequestration: with RGD sequences in the penton base [89], hypervariable regions of hexon, and knob and shaft domains of the fibre playing a role in the Ad5 infection pathway [68], and also being common targets for pre-existing immunity [90]. Pre-existing immunity is a common problem for delivering Ad5 in clinical trials since one can obtain immunogenic epitopes (part of an antigen that can be recognised by the immune system) via either vaccination or natural adenovirus cold infection [90, 91].

To achieve highly efficient targeted delivery with Ad5, both receptor binding (e.g. CAR/integrins) and bridging interaction (e.g. factor X, or FX) are crucial. This FX ‘bridge’ connects the binding with Ad5 hexon with surface-expressed heparin sulphate proteoglycans (HSPG) on hepatocytes. Over-riding both mechanisms was crucial in ablating liver infection in an *in vivo* study [67]. More importantly, studies have reported that in the absence of CAR, HSPG still permits the binding of Ad5 with the assistance of FX [92, 93]. Decreasing liver tropism is a key factor for longer Ad5 circulation in the bloodstream of pre-clinical models, and thereby exploiting the EPR effect for better tumour accumulation [30]. It is important to point out that CAR has no role in liver tropism, which attributes mainly to its larger endothelial gaps (140 nm, which is much greater than the 120 nm for Ad5) and Kupffer cells’ capture [64, 94]. In fact, transduction profile *in vivo* does not correlate with CAR expression, and murine liver with CAR-knock out shows identical tropism as wild-type liver where CAR is present [64, 94]. In addition, a recent study by Xu and colleagues demonstrated the ability

of FX to enhance hepatocyte infection by Ad5 is a result of unexpected ability of FX to protect Ad5 from attachment of IgM's and the classical complement pathway. Interestingly, FX was crucial for infection of the liver of wild-type mice but was not required for such infection in mice that lacked C1q or C4 components. The authors concluded that FX is recruited by Ad5 as a defence mechanism *in vivo*, and this new finding must be taken into account when designing future Ad5 mouse studies [95].

Another barrier to successful systematic administration of Ad5 is the Kupffer cells (KC), which are specialised macrophages located in the lining of liver sinusoid [96]. Alemany *et al.* reported that KC are responsible for 90% of the Ad5 clearance 24 hr after intravenous (i.v.) administration; the co-localisation of fluorescein-labelled Ad5 and KC staining provided strong evidence for this claim. Furthermore, the plasma circulation half-life for Ad5 was less than 2 min [88]. By depleting the KC using clodronate liposomes, Green *et al.* demonstrated the circulation half-life of Ad5 increased by 50% from 2 min to 3 min and improved the dose still circulating at 30-min from 0.8% to 2.5% of the injected dose [97]. Clodronate liposomes destroy KCs by being engulfed by KC, endocytosed and releasing their bisphosphonate cargo into the cytoplasm of the KCs. Bisphosphonate molecules are mistakenly recognised as a cellular pyrophosphate and used by cytosol enzymes to produce a non-hydrolysable ATP analogue. During the TCA cycle within mitochondria, these ATP analogue compounds bind to an ATP-binding site on the ATP/ADP translocase, inhibiting the translocation of actual ATP and ADP molecules and resulting in the complete breakdown of mitochondria. Since mitochondria are the essential energy source within cells, their malfunction results in KC death [98].

Although genetic engineering can be used to modify Ad5 capsid proteins to avoid non-target cell infection and interaction with pre-existing immunity [99], the magnitude and complexity of the changes needed often leads to inefficiencies in viral production and eventual loss of infective capacity [100]. Chemical modification of therapeutic agents can

improve their bloodstream compatibility and ultimately their accumulation at target sites of disease [101, 102]. Such chemical ‘stealth’ has been particularly useful in modifying Ad5. Indeed, the addition of amine-reactive hydrophilic polymers, such as poly-ethylene glycol (PEG) or poly-[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA), provides a simpler, more effective and non-heritable method of modifying Ad5 than genetic engineering [100, 103]. Pearce *et al.* have shown ~1800 free lysine residues to be available on Ad5 capsid for reaction, and these amine functional groups are often a good target for conjugate to PEG or PHPMA [104]. Stealthing Ad5 with PEG or PHPMA can dramatically reduce liver capture and enhance bloodstream circulation and tumour accumulation in murine pre-clinical models [105]. However, stealthing may still not provide sufficient coating of Ad5 to allow effective intravenous administration in humans. In particular, conventional polymers provide little protection of the acidic regions of the Ad5 capsid [106]. Besides avoiding liver tropism due to increase in size and shielding, the advantage of Ad5 chemical modification is to decrease its interaction with bloodstream neutralising antibodies, P selectins (cell adhesion molecules), and complement proteins (from innate immune system) [107]. Here, sections 1.2.2 and 1.2.3 review the current research progress of PEG-coated Ad5 (Ad5–PEG) and PHPMA-coated Ad5 (Ad5–PHPMA).

1.2.2 PEG-coated adenovirus

PEGylation of anti-cancer therapeutic nanoparticles, proteins, and nano-medicines (e.g. liposomes) has been studied for nearly two decades to reduce the antigenicity and immunogenicity of conjugated therapeutics *in vivo*. PEG can be formulated in a range of different molecular weights (MW), and usually the longer the PEG, the more favourable its ability to enhance plasma circulation half-life of the conjugated drugs or therapeutics. PEG creates good steric hindrance at atomic level to better shield these nanoparticles exposing to blood protein and immune system detection [108, 109]. In 1999, O’Riordan and colleagues were the first group to synthesise Ad5–PEG_{5k}, and show that both protection against

neutralisation and maintenance of 45% infection could be achieved. They also demonstrated the surface charge changed from -48 mV for Ad5 to -20 mV for Ad5-PEG_{5k}, but did not measure any change of size produced by this modification [110].

Mok *et al.* also produced Ad5-PEG_{5k} and demonstrated that although the *in vitro* infection had been compromised (decreased CHO 2-hr cell infection by 80%), there was no difference in liver infection between Ad5 and Ad5-PEG. However, the authors showed for the first time that Ad5-PEG significantly decreased the capture by KC compared to Ad5, but interestingly did not reduce liver toxicity (as assessed by liver enzyme release). These finding perhaps indicate that the PEGylation achieved was sufficient to prevent CAR mediated infection *in vitro*, but that hexon regions were poorly protected and so infection of murine hepatocytes *in vivo* remained high. Unfortunately, the paper was published without attempt to quantify any change of Ad5 size or surface charge, so the exact extent of Ad5 surface modification was unknown [111].

Alemaný and colleagues were the first group to report the *in vivo* plasma circulation profile of Ad5-PEG_{5k}, showing that PEGylation enhanced the 30-min plasma circulation from 0.2% for Ad5 to 1.5% for Ad5-PEG. The paper also showed three orders of magnitude decrease in A549 cell transduction *in vitro* with Ad5-PEG (measured by GFP transgene expression). Although size was not reported, the measured zeta potentials for Ad5 and Ad5-PEG were -10 mV and -6 mV, respectively [88].

Eto *et al.* were the first group to report the actual size change: from 122 nm for Ad5 to 135 nm for Ad5-PEG_{5k}. Notably, the group attached peptide sequence RGD (peptide motif in Ad5 penton that interacts with host cell's membrane integrins) at the tip of the PEG and compared the transduction efficiencies among Ad5, Ad5-PEG, and Ad5-PEG-RGD. While Ad5-PEG reduced the *in vitro* infection of A549 cells by 200-fold compared to un-modified Ad5, Ad5-PEG-RGD was able to return infection back to the same level as that of control

Ad5 [112]. However, there is a high concern regarding whether this design could be of benefit in *in vivo* studies or clinical trials since the highly exposed RGD motif can easily interact with membrane integrins in liver. In fact, since the publication of this paper in 2005, the same group has yet to publish any subsequent paper on the *in vivo* testing of Ad5-PEG-RGD.

Gao and colleagues were the first group to report a complete bio-distribution study of Ad5-PEG_{5k} and a subsequent efficacy study. The sizes reported for Ad5 and Ad5-PEG were 113 nm and 138 nm, respectively. BALB/c mice were implanted with HEK 293 tumours, and the experiments were performed when tumours reached 100 to 150 mm³. 30 min after i.v. administrated 1×10^{10} v.p. per mouse, 1% of Ad5 and 4% of Ad5-PEG were detected in plasma (n = 5), showing for the first time PEGylation of Ad5 could improve plasma circulation. Tumour growth was monitored for the remaining mice (n = 8) for 19 days. At two weeks after the treatment, the tumour volume of Ad5-PEG-treated mice was significantly smaller than Ad5-treated mice; 50% reduction in tumour volumes was achieved at day 19 when all the mice were culled. No survival data were shown in this study. The authors argue the higher Ad5-PEG tumour accumulation and better efficacy were the result of the EPR effect due to better circulation of Ad5-PEG in mouse plasma. However, it is notable that this study did not use a strategy to allow shedding of the PEG coat within the tumour, so the mechanism and level of tumour cell infection are unclear. [113].

Lastly, unlike most of PEGylation studies where 5k-PEGs were used, Doronin *et al.* were the first group to attach 20k-PEG to Ad5 and to directly compare the bio-distribution effects of Ad5-PEG_{5k} and Ad5-PEG_{20k}. The measured sizes for Ad5, Ad5-PEG_{5k}, and Ad5-PEG_{20k} were 110 nm (PDI 0.063), 123 nm (PDI 0.173), and 139 nm (PDI 0.122), respectively, showing for the first time that higher molecular weight PEG resulted in larger Ad5/PEG complexes. This was also the only Ad5-PEG study where polydispersity index values (PDI) were reported for each Ad5 modification. Nude mice bearing Hep3B tumours were injected intravenously with 5×10^{10} v.p. per mouse. At tumour sizes ranged 200 to 400 mm³, systemic

administration of Ad5 and the two Ad5-PEGs showed that Ad5-PEG_{20k} lowered the liver transgene expression by two orders of magnitude at days 1 and 3, while Ad5-PEG_{5k} showed no difference in liver transgene expression compared to Ad5. Due to these findings, Ad5-PEG_{5k} was not included in the survival study. Although there was no significant difference in tumour transgene expression between Ad5 and Ad5-PEG_{20k}, the tumour growth rate was significantly retarded in Ad5-PEG_{20k} mice compared to Ad5-treated mice or PBS-treated mice. The median survival time for mice treated with PBS, Ad5, and Ad5-PEG_{20k} were 14 days, 18 days, and 31 days, respectively. Ad5 alone only improved the efficacy marginally with $p > 0.05$, while Ad5-PEG_{20k} significantly improved the efficacy with $p < 0.001$ [103].

Notably, although many of the Ad5-PEG papers investigated the biological effects, they did not report the characterisation of the chemical modification of the Ad5 capsid. Changes in size and zeta potential have rarely been reported, and the percentage of the Ad5 capsid modified with PEG was never quantified except in Gao's paper [113]. Only two groups (O'Riordan [110] and Gao [113]) used SDS-PAGE to demonstrate the modifications of Ad5 hexon, penton, and fibres by PEG. In addition, many of these earlier Ad5-PEG studies were performed before or around the same time as the publications regarding FX-mediated cellular binding and the interaction between human erythrocytes and Ad5, so very little work has been done to investigate the interaction of Ad5-PEG with FX and human red blood cells. In fact, none of published literature has reported these studies yet. These are important questions that will all be addressed in this thesis.

1.2.3 PHPMA-coated adenovirus

Since the early 1980's, PHPMA has been used to coat various biological compounds and therapeutics, such as antibodies [114], oligopeptides [115], and melphalan (a chemotherapy drug) [116]. However, it was not until 2001, that Fisher and colleagues first coated Ad5 with PHPMA [117]. Unlike PEG which provides a single-point attachment to

Ad5's surface, PHPMA molecules are usually synthesised in a way that provides multiple amine reactive groups, such as 4-nitrophenoxy [117] and thiazolidine-2-thione [106].

Ad5–PHPMA synthesised by Fisher *et al.* increased the viral size from 105 nm to 128 nm, widened the PDI from 0.02 to 0.2, decreased the surface zeta potential from -25 mV to 0 mV, and decreased the band intensity of the 62kDa fibre on Western blot. *In vitro* knockdown of native tropism was demonstrated in A549 cells. The authors then retargeted Ad5–PHPMA with VEGF; with a well-designed experiment to test selectivity, HUVE cells (low CAR, high VEGFR) and SUIT2 cells (high CAR, low VEGFR) were mixed and infected by either Ad5 or Ad5–PHPMA-VEGF. Ad5 showed high transgene expression in SUIT2 cells but relatively low infectivity in HUVE cells, while Ad5–PHPMA-VEGF significantly increased the transgene expression in HUVE cells but not in SUIT2 cells. These results indicated that although Ad5–PHPMA could decrease overall infection, a strong selectivity of infection could be re-stored by retargeting Ad5–PHPMA with specific ligands such as VEGF [117].

The same research group, Green *et al.*, also became first group to perform *in vivo* studies on Ad5–PHPMA to characterise its pharmacokinetics, liver capture, and toxicity. Systemic administration of between 10^9 and 10^{11} copies of Ad5 or Ad5–PHPMA per BALB/c was performed into mice bearing no tumour. The authors showed that the viral dose still circulating at 30 min increased: (i) from 0.1% for Ad5 to 0.5% for Ad5–PHPMA for mice received 10^9 v.p.; (ii) from 3% for Ad5 to 50% for Ad5–PHPMA for mice received 10^{11} v.p. In addition, after integrating the area under the curve (AUC) for both circulation profiles, AUC of Ad5–PHPMA was 3.5 larger than AUC of Ad5 with significant p-value < 0.01 . The paper showed for the first time that the higher the initial administered viral dose, the higher the liver saturation rate, resulting a better plasma circulation profile. In addition, compared to Ad5, Ad5–PHPMA gave an at least 100-fold decreased hepatic transgene expression at 24 hr at all doses and showed no elevated level of plasma alanine aminotransferase (ALT), a measurement of liver toxicity [118]. The decrease in hepatic infection seen with PHPMA

which has never been reported using PEGylation, may reflect the superior ability of PHPMA to coat the hexon protein.

Four years later, Green and colleagues published a new paper reporting the pharmacokinetics and bio-distribution of Ad5, Ad5-PHPMA, and Ad5-PHPMA-FGF in AB22 tumour bearing mice. It is important to note that AB22 are a murine cell line low in CAR but rich in FGF membrane receptors, so the authors hoped to specifically target the Ad5-PHPMA-FGF to the tumour. *In vitro* data showed that Ad5-PHPMA decreased the infectivity by 20-fold, whereas Ad5-PHPMA-FGF was able to restore the infection back to the level of un-modified Ad5. In the *in vivo* study, 30 min after the v.p. injection, the percentages of Ad5, Ad5-PHPMA, and Ad5-PHPMA-FGF still circulating in plasma were 1%, 12%, and 21%. Although the plasma circulation of Ad5-PHPMA-FGF was impressive, compared to Ad5, the transgene expression levels mediated by Ad5-PHPMA-FGF were greatly decreased in all organs (livers, tumours, spleen, kidneys, lungs, and heart); in particular, the transgene expression in the liver dropped by four orders of magnitude. On the other hand, Ad5-PHPMA again showed much lower transgene expression in all organs except tumour, where a 40-fold higher v.p. accumulation were detected. The authors later discovered the good plasma circulation of Ad5-PHPMA-FGF but poor tropism to tumour and other organs was due to its binding to murine erythrocytes, perhaps mediated by non-specific charge interactions. Although erythrocyte do not express FGF receptors, their net negative surface charge attracts the overall net positive charged of FGF molecules. The EPR effect and a correlation between plasma circulation and tumour accumulation did not apply in this case because Ad5-PHPMA-FGF needed to first dissociate from the red blood cells before it could enter through the endothelial gaps of tumour, which are smaller than the size of erythrocytes. This study provided great insight for future retargeting strategies indicating that the use of a ligand that has neutral charge or the use of no ligand at all (as seen in this study that Ad5-PHPMA was the best candidate) was a good approach. It also demonstrated that pre-testing all

the possible blood factors that could potentially interact with the Ad5–PHPMA was an important validation step [119].

In their most recent survival study in 2012, Green *et al.* demonstrated by pre-injecting clodronate liposomes (to suppress KC) into the mice 24 hr before the v.p. administration and pre-dosing the v.p. into the liver, that 5% of Ad5 and 90% of Ad5–PHPMA could be recovered in plasma circulation 30 min after v.p. administration. The paper also showed for the first time a strong correlation between AUC of circulation profile and v.p. tumour accumulation ($R^2 = 0.7149$). Five doses (injected around every 4 days) of 2×10^{10} Ad5–PHPMA were administered per nude mouse bearing HepG2 xenograft at size 10 to 20 mm³, the control mice received the same time-point treatment with only PBS. After one week, the tumours of mice treated with Ad5–PHPMA were maintained at around 30 mm³ and subsequently maintained this size for the remainder of the study, while the tumours of mice treated with PBS grew exponentially up to 600 mm³ at day 40 when all the mice were culled. This efficacy study showed significant improvement ($p < 0.001$) in the survival of mice treated with Ad5–PHPMA. However, the data relating to the long-term survival study of mice treated with Ad5 alone versus Ad5–PHPMA were not reported [97].

Lastly, conventional polymers provide little protection of the acidic regions of the Ad5 capsid. To improve stealthing of these regions, Subr *et al.* further added quaternary amine groups (QA) to the PHPMA side chain, making PHPMA positively charged which increased its electrostatic binding to the negatively charged surface of Ad5 [106]. However, this inclusion can result in such a dense coating that Ad5 infection activity cannot be fully recovered; as a result, a reducible disulphide bond (SS) was introduced to the linkage between Ad5 and PHPMA and of Ad5 and QA. Subr and colleagues tested the *in vitro* protection against antibody neutralisation and human erythrocyte binding between Ad5, Ad5–SS–PHPMA, QA–Ad5–SS–PHPMA, and QA–SS–Ad5–SS–PHPMA. SDS-PAGE results showed that both QA and PHPMA could be successfully reduced by β -mercaptoethanol (BME) [106].

In the absence of reducing buffer, the penton and fibre bands disappeared in all three modifications while the hexon band showed an apparent decrease in intensity; in the presence of reducing buffer, the hexon, penton, and fibre bands were stained at the same level as control Ad5. This suggested a good coating of penton and fibre was achieved, but the coating of hexons was incomplete for all three modifications [106]. All three Ad5 modifications were able to lower human erythrocyte binding from 100% to 5%, demonstrating that PHPMA alone provided a good coating against erythrocyte binding without the assistance of QA. While, Ad5–SS–PHPMA was able to decrease the binding to anti-Ad5 antibodies from 100% down to 60% ($p < 0.05$), both QA–Ad5–SS–PHPMA and QA–SS–Ad5–SS–PHPMA were able to lower its antibody binding down to 25% ($p < 0.001$) [106]. Similar data were observed in the FX binding experiments using low-CAR SKOV-3 cells: The presence of FX was able to increase the reporter gene expression of Ad5, Ad5–SS–PHPMA, QA–Ad5–SS–PHPMA, and QA–SS–Ad5–SS–PHPMA by 58 fold, 27 fold, 0 fold, and 0 fold. These results suggest that QA significantly improved protection of Ad5 to above the level of coating achieved with conventional PHPMA [106]. Whether this promising reducible design of Ad5–PHPMA was applicable to *in vivo* study for better tumour accumulation and lower liver capture has yet to be published. The fact that QA–SS–Ad5–SS–PHPMA could block the binding of FX was an auspicious step towards clinical application.

1.3 Introduction to therapeutic ultrasound

Therapeutic ultrasound may be considered to be any application of ultrasound which instigates a direct improvement in disease outcome or enhances the activity of a co-applied conventional therapy. A huge range of exposure conditions and applications are therefore covered by this definition. These include the use of high intensity focussed ultrasound (HIFU) for thermal ablation of diseased tissue [120, 121], cataract treatment by phacoemulsification [122], and the break-down of calculi such as kidney stones and gall stones so that they can be passed from the body, a process known as lithotripsy [123]. In addition, low-intensity

ultrasound has been used for stimulating tissue and bone repair [124] and to reversibly disrupt the blood-brain barrier [125]. Therapeutic ultrasound is also known to enable targeted drug delivery [126, 127] and localised enhancement of drug activity for applications ranging from sonothrombolysis for stroke therapy [128] to cancer therapy [127]. It is this final application and the underlying therapeutic ultrasound mechanisms responsible that will be the focus of this section.

Ultrasound is the oscillation between compressional (high) and rarefactional (low) pressure fluctuations with a frequency greater than 20 kHz. Therapeutic ultrasound tends to use frequencies in the range 0.5 to 5 MHz [120]. A key advantage of using therapeutic ultrasound is its ability to exert a localised effect from the surface of the skin up to 15-20 centimetres into the body. Such non-invasive and targeted application contrasts markedly with surgical intervention and chemo- and radiotherapy which have a high potential to damage neighbouring healthy cells. Lower-frequency ultrasound waves give a greater penetration depth in soft tissue; for instance, 1 MHz ultrasound waves will propagate to more than 12 cm within the body, whilst 4 MHz waves will become heavily attenuated within 3 cm. In addition, increasing frequency results in decreasing focal volume [120].

Depending on the exposure conditions used (e.g. frequency, pressure, duration) ultrasound can instigate two different categories of effects that can be used to enhance drug delivery: thermal and mechanical [129-132].

Thermal effects arise from the absorption of the sound waves by tissue and can be used to achieve temperature increases that are moderate enough to cause no direct tissue damage but are sufficient to promote release of a drug from thermoresponsive carrier systems [126, 127].

Mechanical effects are instigated by several mechanisms. Cavitation effects are produced by microbubbles within the target tissue [129, 131]. These may be spontaneously

nucleated by ultrasound or may be introduced by injection (e.g. in the form of ultrasound contrast agents (UCAs) [133]). Originally developed to enhance contrast in diagnostic ultrasound imaging, UCAs are now being applied to provide ‘cavitation nuclei.’ These inert gas-filled bubbles are of a diameter ranging from 0.5 to 10 μm , and are coated to prevent bubble dissolution and thereby improve stability *in vivo* [134]. However, the half-lives of these microbubbles within the human bloodstream are around 6 minutes after intravenous administration [135, 136], indicating multiple injections may be required for sustainable inertial cavitation in *in vivo* experiments. There are many commercially available and biodegradable microbubbles, including Albunex® (air in albumin shell), Definity® (perfluoropropane in lipid shell), Imagent® (perfluoropropane in lipid shell), Levovist® (air inside galactose shell), Optison® (perfluoropropane inside albumin shell), Sonazoid® (perfluorobutane inside lipid shell), and SonoVue® (sulphur hexafluoride inside lipid shell). Importantly, because UCAs are very strong scatterers of ultrasound, they can be detected and mapped non-invasively using conventional B-mode ultrasound, even before they are excited using therapeutic levels of ultrasound for drug delivery [137, 138].

The response of microbubbles to acoustic excitation is termed acoustic cavitation, and two qualitatively different behaviours can be discerned: inertial (also referred to as transient) and stable (also known as non-inertial) cavitation [127, 139]. Non-inertial cavitation (**Figure 1-8A**) occurs when bubbles oscillate linearly or non-linearly about their equilibrium radius over several acoustic cycles repeatedly. The rarefaction phase of an ultrasound wave causes the microbubbles to expand, whilst the compression phase makes the microbubbles contract. Stable cavitation has been found to result in microstreaming, i.e. enhanced momentum transfer to the surrounding liquid that can enable the microstreaming of drugs [127, 140].

Inertial cavitation (**Figure 1-8B**) occurs when the pressure amplitude becomes sufficiently large to initiate rapid bubble growth during the rarefactional half-cycle of the ultrasound wave, resulting in microbubble expansion typically greater than a factor of two;

this growth is followed by violent collapse during the compressional half-cycle, under the effect of the inertia of the surrounding liquid [127]. Inertial cavitation may generate heat, free radicals, shock waves and shear forces due to surrounding fluid motion [141]. The last of these is believed to be the main mechanism involved in delivering therapeutic agents deep into solid tumours [142].

To qualitatively determine if microbubbles are experiencing stable or inertial cavitation, a Fast Fourier Transform (FFT) of the acoustic emissions from the bubbles can be performed. **Figure 1-8C** represents a typical frequency spectrum for both stable (green line) and inertial (blue line) cavitation. While stable cavitation produces only detectable signals at harmonic and subharmonic frequencies (e.g. $F_0/2$, $F_0/3$), inertial cavitation produces broadband emissions.

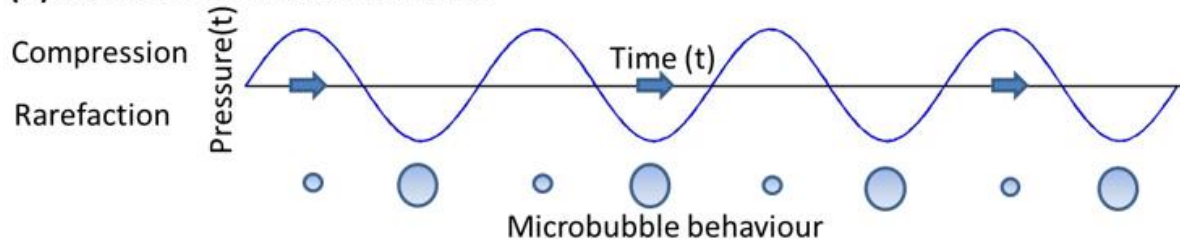
Another important mechanical effect is acoustic radiation force, which is produced by momentum transfer from the ultrasound wave as it propagates through tissue [143]. This tends to act to translate particles away from the ultrasound transducer and can thereby enhance extravasation, convection, and diffusion through a tumour matrix [131, 132] thus serving as a powerful mean to drive drugs and small molecules from blood vessels into solid tumours.

The majority of ultrasound-enhanced drug delivery research during the twentieth century has been performed *in vitro* using cell and tissue cultures. Since then a growing number of studies have reported the beneficial effects of ultrasound-enhanced drug delivery *in vivo*, demonstrating targeted release, improved delivery, and enhanced extravasation of therapeutic agents into solid tumours.

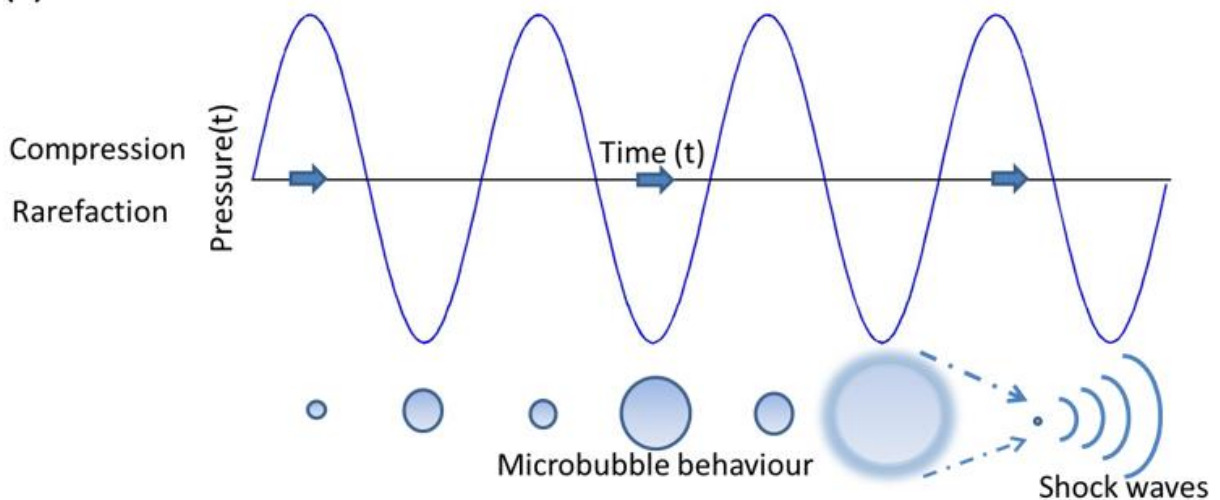
Sections 1.3.1 and 1.3.2 will discuss the biological effects of ultrasound in the tumour environment: sonoporation, convection, and extravasation of drugs. Cavitation effects and radiation force are the main driving forces behind these. Section 1.4 reviews various

ultrasound-enhanced drug delivery vectors and the different types of drugs encapsulated within such vectors.

(A) Stable or non-inertial cavitation



(B) Transient or inertial cavitation



(C) Analysis of stable and inertial cavitations by frequency-FFT graph

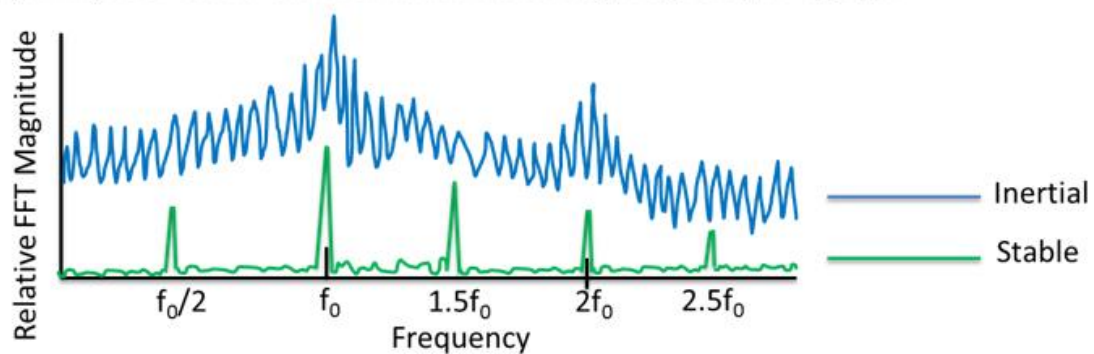


Figure 1-8: Schematic diagram illustrating the effects of acoustic fields of identical frequency but differing pressure on microbubble behaviour. (A) Under stable cavitation with lower pressure, microbubbles oscillate periodically between small and relatively larger size under compression and rarefaction, respectively. (B) In the case of inertial cavitation, the higher pressure causes microbubbles to collapse very rapidly to a small fraction of their initial volume, producing shock waves. (C) After transforming the microbubble cavitation data from raw voltage traces via Fast Fourier Transform (FFT) algorithm, a frequency-FFT graph can be plotted: while stable cavitation shows only detectable signals at subharmonic frequencies (e.g. $F_0/2$) and their multiples, inertial cavitation shows detectable signals through the entire frequency domain. Under the same pressure, FFT magnitude is usually higher for inertial cavitation than stable cavitation.

1.3.1 Sonoporation

A mechanism for ultrasound-enhanced increased gene uptake to tumour cells is sonoporation, a process in which ultrasound is used to alter the permeability of the cell plasma membrane [144, 145]; collapsing bubbles are believed to create transient holes in the cell membrane, through which large molecules can enter cells [127, 144]. Sonoporation can provide a very specific and high concentration of delivered drug at the site of interest while minimizing the overall exposure of the rest of the body to the drug [145]. It may be of particular advantage for the delivery of free nucleotide which would otherwise have limited passage across the plasma membrane due to its large size and net negative charge. However, a consensus definition of sonoporation has not been well established in the scientific community. Should the definition include an exchange of intracellular and extracellular fluids, or simply a few open pores on the cell membrane? How is permeability of such phenomenon measured in real time? How does one define a ‘successful’ sonoporation? These are all questions that needed to be addressed before this phenomenon can be firmly established.

Several recent studies have tried to measure the cell membrane pore sizes opened under ultrasonic sonoporation by scanning electron microscope (SEM) imaging. These reports have all used commercially available microbubbles as cavitation nuclei and produced measurements of ~75 nm in rat mammary carcinoma cells exposed to 1.15 MHz ultrasound (400 kPa and 10 sec) [146], ~56 nm in DU 145 prostate-cancer cells exposed to 24 kHz ultrasound (700 kPa, 10% duty cycle, and 20 acoustic pulses) [147], and 110 ± 40 nm in *Xenopus laevis* oocytes exposed to 1 MHz ultrasound (300 kPa and 0.2 sec) [148]. Even in the absence of microbubbles or liposomes, ultrasound alone still increased the drug or gene delivery to target tumour cells at 1 MHz [149, 150] in *in vitro* experiments, and one possible explanation is that there are still adequate levels of dissolved gas in the experimental cell media solution to allow cavitation activity. This raises concerns regarding the clinical applicability of such effects as the natural efficient removal of bubbles from the circulatory

system means that there may not be a sufficient supply of cavitation nuclei [142]. In addition, the preparation procedures of SEM to image cells is known to create some pores and/or other artifacts on cell membrane [151], so whether these observed pore sizes are due to sonoporation or SEM is still unclear.

The micron sized cavitation agents required for sonoporation will have size-restricted direct contact with tumour cells (see section 1.4.1) and so may only impact upon the tumour associated vasculature. Hence, in the context of drug delivery to solid tumours, sonoporation may ultimately be most effective in enhancing strategies that seek to destroy tumours by killing their supporting endothelial cells.

1.3.2 Enhanced extravasation and convection

For the purpose of this literature review, convection is defined as the collective movement of fluid (e.g. blood plasma and interstitial fluid) within tumour vasculature and tissue, whereas extravasation refers to the transport of therapeutic agents from the bloodstream into the surrounding tumour tissue.

There are two mechanisms by which ultrasound may increase the convection of liquid. First, acoustic streaming produced by an external ultrasound source may instigate flow of fluid in the direction of the sound propagation [127, 152]. The second mechanism of increased convection is known as microstreaming in which stable or inertial cavitation creates shear flow in the surrounding liquid [140, 152].

Stieger *et al.* demonstrated that convection (resulting from 1.00 and 2.25 MHz ultrasound exposure with microbubbles) is the dominant transport mechanism enhancing vascular permeability and delivery of therapeutic agents in a chorioallantoic membrane model for *in vivo* visualization of these ultrasound effects on blood vessels. Lipid-shelled perfluropropane-filled microbubbles and FITC-labelled dextrans were co-administrated into

the vascular system of the embryo. The study suggested the threshold pressure for extravasation was 0.5 MPa for 1 MHz and 1.6 MPa for 2.25 MHz ultrasound; the remaining ultrasound parameters for both frequencies were 10-cycle pulses at a PRF of 500 Hz over 5 sec. The control group (with no ultrasound exposure) had no sign of extravasation of the labelled dextran and showed close to zero flow velocity, whereas the flow rates of dextran inside the vasculature were increased up to $188 \pm 75 \mu\text{m/s}$ and $362 \pm 150 \mu\text{m/s}$ by 1 MHz and 2.25 MHz exposure, respectively. The precise role and importance of the microbubbles in mediating these effects is hard to gauge as the influence of these exposures on this model were not tested in their absence [153]. Arvanitis *et al.* utilized quantitative passive cavitation detection techniques to demonstrate that the correlation between inertial cavitation and the extravasation of drugs [152]. Several recent studies have also demonstrated that drugs exposed to ultrasound in the presence of microbubbles achieve greatly enhanced extravasation into solid tumours [154-158].

Attempts to better understand and control extravasation mechanisms were recently made by Bazan-Peregrino *et al.* [159]. A phantom vessel running through agar gel containing breast cancer cells was developed as an *in vitro* 3D tumour model for these studies. Oncolytic adenovirus Ad5EHE2F-Luc was introduced through the phantom vessel, and a focused 0.5 MHz HIFU transducer was used to instigate acoustic cavitation in the presence and absence of UCAs. While delivering the same amount of acoustic energy, the ultrasound exposure parameters were adjusted to maximise either the ultraharmonic content (360 kPa, 90% duty cycle and PRF of 10 Hz for stable cavitation) or broadband spectral content (1.25 MPa, 6.5% duty cycle and PRF 10 Hz for inertial cavitation) of the acoustic emissions [159].

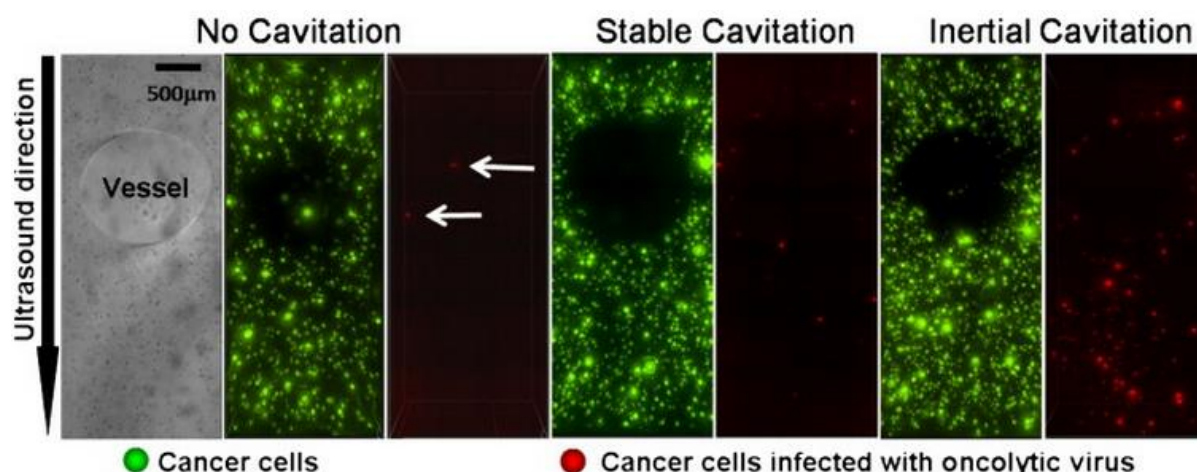


Figure 1-9: Extravasation of adenovirus with the assistance of focused ultrasound and microbubbles. BT-474 cells (carrying green fluorescence protein, shown as green dots above) were embedded in tumour-mimicking model containing a vessel. Co-administration of SonoVue® microbubbles and adenovirus (carrying red fluorescence protein, shown as red dots above) was performed through the flow vessel while 0.5 MHz ultrasound applied at 0 MPa (no cavitation), 360 kPa (stable cavitation), and 1.25 MPa (inertial cavitation). Extravasation of the oncolytic virus is greatly enhanced in the presence of stable and in particular inertial cavitation, with much of the enhancement being observed in the direction of sound propagation; reproduced from [159] with permission of *Journal of Controlled Release*.

In the absence of ultrasound, very few cancer cells (naturally expressed green fluorescence proteins and shown as green dots in **Figure 1-9**) were successfully infected by the oncolytic virus (genetically engineered with red fluorescence proteins and shown as red dots in **Figure 1-9**). However, the instigation of cavitation correlated with increased adenovirus extravasation resulting in increased infection efficacy. Interestingly small increases in the total amount of adenovirus transferred into the agar led to much more marked increases in the transgene expression achieved. Stable cavitation caused a two-fold increase in the number of viral particles extravasated, which translated into a 10-fold increase in expression at 24 hr. Yet more impressively inertial cavitation caused a four-fold increase in viral concentration which translated into a 200-fold increase in luciferase expression, with much of the increase being observed in the direction of sound propagation (**Figure 1-9**). This indicates the key role that therapeutic ultrasound in general and inertial cavitation in particular can play in improving both the level of therapeutic agent transferred and the distribution of the agent throughout the target tumour [159]. Such findings have since been shown to be applicable to the *in vivo* setting [45].

1.4 Encapsulation of drugs for therapeutic ultrasound

Ultrasound has been used to trigger the release of anti-cancer drugs from delivery vectors including liposomes, polymeric microspheres and self-assembled polymeric constructs [127, 142]. This release has been achieved using the mechanical or thermal effects of ultrasound. Mechanical effects have been exploited to release micelle-encapsulated doxorubicin [160], liposome-encapsulated thrombin [161], and nano-emulsions [162], while thermal effects have been used to trigger doxorubicin release from temperature-sensitive liposomes [126, 127].

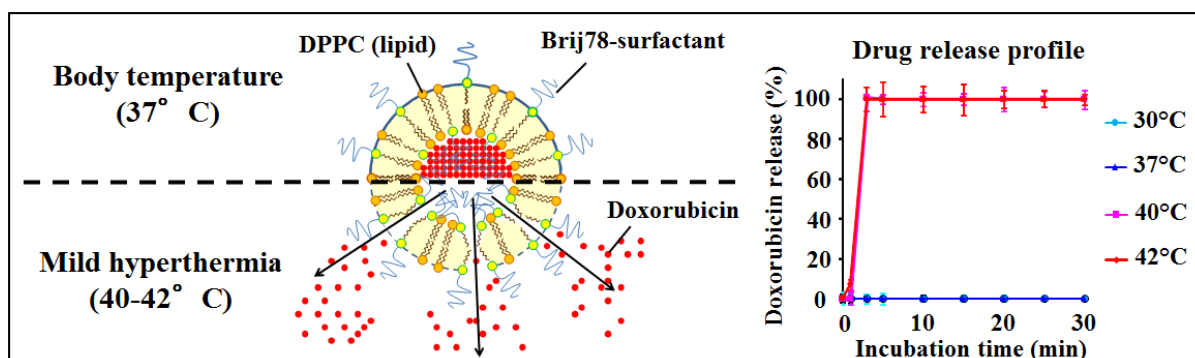
1.4.1 Ultrasound-enhanced delivery vehicles

Many studies have attempted to co-administer cavitation nuclei and non-encapsulated therapeutics (such as drugs or genes) and then apply therapeutic ultrasound [127, 158, 159, 161, 163-168]. Such a strategy can result in the temporary formation of pores in target cell membranes through which therapeutics can achieve efficient passage into the cell. Despite showing promise *in vitro* [144, 145], this ‘sonoporation’ method (discussed in section 3.1) is limited *in vivo* by the non-specific interaction of therapeutics with non-target tissues and the requirement for the therapeutic to co-localise with the agent used as a cavitation nuclei. The applicability of sonoporation has also been limited by poor transfection efficiencies, which have in general not exceeded 3% of the target tissue volume, resulting in limited therapeutic effect [169]. To reduce non-specific interactions and improve the co-localisation of the therapeutic and the cavitation nuclei, therapeutics have been incorporated into vectors designed to have intrinsic sonosensitivity, i.e. possess the ability to undergo inertial cavitation in response to ultrasound. A range of ‘all-in-one’ acoustically active delivery vectors including microbubbles, liposomes, and nanobubbles are now under development [127, 131, 167].

Traditionally, used solely as ultrasound contrast agents (UCA), microbubbles have more recently been formulated to serve as both delivery vector and cavitation nuclei [161, 163-166]. Therapeutic agents can be loaded inside microbubbles, within the microbubble shell, or can be conjugated directly to the surface of the shell. Under ultrasound-induced cavitation, these microbubbles are designed to collapse and release entrapped therapeutic agents within target tumours [127, 165, 167]. However, microbubbles do not readily extravasate into tumours due to their relatively large diameter which is usually in the range of 0.5 to 10 μm , with a mean diameter of about 2.5 μm [135]. As the gaps between neighbouring tumour associated endothelial cells have been calculated to be between 400 and 600 nm [27] or 100 and 1000 nm [28], the vast majority of micron sized bubbles will be prevented from extravasating through these gaps and gaining access to the tumour. Once through the endothelial gaps, therapeutics still need to penetrate through the tumour interstitium, a high pressure environment with dense extracellular matrix and intercellular spaces which have a mean diameter of 1.7 μm and range from 0.3 to 4.7 μm [26]. It is also apparent that many of these formulations are cleared rapidly from the bloodstream due to poor stability and capture by the reticuloendothelial system (RES) and entrapment in the capillary beds of the lung and spleen [170, 171]. Their micrometre size and poor pharmacokinetics may therefore ultimately limit the ability of microbubble formulations to benefit from the enhanced permeability retention effect (EPR) [172, 173] and achieve enhanced passive accumulation in tumours. In response to these limitations, smaller and more robust sonosensitive delivery vehicles (e.g. liposomes and nanobubbles) are being developed.

Liposomes are vesicles comprised of a bilayer of either natural or synthetic phospholipids. Usually therapeutic agents are entrapped inside the liposome for stability and the external layer of the liposome is frequently coated with tumour-cell targeting ligands and/or protective polymer to minimise recognition by the RES [126, 161, 174]. Different types of liposomes have different sizes, but those used clinically usually measure between 100

nm and 400 nm [175]. Initially, ultrasound-enhanced liposome-delivery experiments were performed in the presence of ultrasound contrast agents such as microbubbles [176]. More recently, there have been reports of liposomal formulations that can achieve triggered release in response to the thermal [6, 15] or mechanical effects of ultrasound [175, 177-181] even in the absence of microbubbles.



Figures 1-10: Doxorubicin release from thermosensitive liposomes. Thermosensitive liposomes stably entrap doxorubicin at physiological temperature, and upon exposure to mild hyperthermia (4 to 5°C temperature elevation) by external and localised sources (e.g. ultrasound, light, microwave), phospholipid bilayers of liposomes disintegrate and release drug content; reproduced from [182] with permission of *Journal of Controlled Release*.

Thermosensitive liposomes (TSL) were first synthesized by Weinstein *et al.* in 1980 to treat to treat solid L1210 xenograft tumours in mice [183], but it was not until 2007 that Dromi *et al.* demonstrated therapeutic ultrasound can serve as a source of hyperthermia and trigger doxorubicin release from these formulations [126]. Control non-thermosensitive liposomes (NTSL) do not release entrapped drugs at physiologic or raised temperatures, whereas most TSLs are designed to release entrapped drugs only upon exposure to a temperature of 42°C or higher [184]. The sensitivity of TSLs to slight and non-lethal temperature elevation of around 4 to 5°C, does not therefore require a continuous ultrasound exposure to achieve a traditional ‘ablation’ effect [121]; instead, non-continuous ultrasound with low duty cycle (10%), pulse repetition frequency (1 Hz), and 120 pulses can be used [126, 185]. A schematic representation of how doxorubicin release occurs from thermosensitive liposomes is provided in **Figure 1-10**.

Where mechanical response is concerned, the recent work of Evjen *et al.* [177, 178] is of particular interest and has improved the understanding of how phospholipid composition can be modulated to enhance sensitivity to mechanical disruption. However, the ultrasound exposure parameters (40 kHz, 100% duty cycle, up to 6 min) used in these reports are unlikely to be clinically applicable and ultimately mechanically triggered release will require the inclusion of gas to provide cavitation nuclei. Indeed, Huang *et al.* have suggested that most liposome preparations can actually entrap gas to a certain degree if freeze-dried phospholipid cakes are formulated in the presence of mannitol under vacuum pressure of 100 torr. One hypothesis for this process is that defects in the liposomal bilayer created during the lyophilisation process expose hydrophobic surfaces which, upon re-suspension in buffer, allow contact with and entrapment of air [180]. Huang *et al.* later used this procedure to formulate 780 ± 70 nm calcein-containing liposomes comprised of egg PC, DPPE, DPPG, and CH. These liposomes were reported to contain a payload of one-third gas and two-thirds drug in aqueous solution and could be acoustically triggered *in vitro* by applying 1 MHz ultrasound at 2 W/cm^2 for 10 sec [181]. Studies have revealed considerable heterogeneity in the samples prepared by this method, with substantial numbers of liposomes having diameters exceeding $1 \mu\text{m}$ [180].

Several studies have reported the use of empty liposomal bubbles as ultrasound contrast agents without entrapping any therapeutic agents [186-188]. Suzuki *et al.* reported the preparation of liposomal bubbles with a mean diameter of $1 \mu\text{m} \pm 300 \text{ nm}$ as measured by dynamic light scattering. These were injected intraperitoneally in combination with a non-encapsulated luciferase reporter gene plasmid into mice implanted with S-180 cells while 1 MHz, 1.0 W/cm^2 ultrasound was applied transdermally for 1 min and 50% duty cycle. The luciferase signal from the transfected cells was two orders of magnitude higher when plasmid was combined with liposomal bubbles and ultrasound than when combined with liposomal

bubbles only. It is unclear from these studies how such an ultrasound exposure would have benefitted transduction with this plasmid in the absence of the bubbles [189].

Kheirrolomoom *et al.* combined the advantages of both liposomes and microbubbles by successfully conjugating liposomes to the microbubble shell. This 1.5- μm liposome-microbubble hybrid oscillated in a similar manner to control microbubbles in response to a single 200 kPa, 2.25 MHz acoustic pulse and could successfully deliver fluorescent cholesterol to a monolayer of PC3 cells. After microbubble oscillation or disruption, the liposomes were detached from the microbubbles, but the study did not characterise the liposome release profile. While such a strategy should enable the micron sized bubble-liposome formulation to release drug within tumour vasculature, thereby allowing penetration of free drug into the tumour, at a size of 1.5- μm this formulation will not be able to benefit from the raised passive tumour accumulation provided by the EPR effect [190].

A recent innovation is the creation of nanobubble cavitation nuclei, which are echogenic and small enough to passively and actively extravasate through the gaps in the tumour associated endothelium [154, 191, 192]. There are currently two types of nanobubbles under development. Free nanobubbles [154, 155] and nanobubbles trapped on the surface of solid nanoparticles [191, 192]. Wang *et al.* prepared coumarin-6 and sulphur hexafluoride loaded lipid nanobubbles comprised of tween-80, cholesterol, and soybean lipid with a reported diameter of 300 nm. To date there are no reports of the combined use of these nanobubbles with ultrasound to instigate drug delivery [155]. In similar studies, Gao *et al.* synthesized perfluoropentane 300 to 800-nm nanobubbles with doxorubicin as a payload, a formulation which demonstrated a few interesting and potentially useful properties. In *in vitro* tests, although these nanoemulsions were very stable at room temperature, upon heating to physiological temperature they fused to form micron-size bubbles. Provided this can be achieved over the desired timescale, and such fusion does not occur immediately post injection, this may allow nano-sized bubbles to accumulate within the tumour by EPR and

then fuse and expand to become micron scale and ultrasound responsive [154]. Indeed in studies using these agents, Rapoport *et al.* showed that such a nano to micro scale conversion was detected within one minute following direct intratumoural injection and even following intravenous injection into mice with tumors of 2000 mm³. Although such conversion could not be detected in smaller tumours (60 mm³), impressive retardation of tumor growth was instigated by the use of nano-bubbles in combination with ultrasound in mice with 100-150 mm³ tumors [162].

The second type of nanobubble, in which gas is entrapped on the hydrophobic surface of solid nanoparticles, is also a notable development. Wagstaffe *et al.* recently used this approach to demonstrate that LUDOX®-coated polystyrene nanoparticles with rough surface morphology can significantly lower the threshold for cavitation, i.e. the minimum pressure required for ultrasound to instigate inertial cavitation. LUDOX® is a commercially available silicon oxide of 15 to 25 nm in diameter, and the LUDOX®-coated nanoparticles produced were in the size range of 300 nm to 600 nm. In *in vitro* tests these rough surfaced nanoparticles lowered the cavitation threshold of water from 5 MPa to less than 0.5 MPa [192], and should this formulation prove to be compatible with *in vivo* delivery it may represent a significant step forward in providing cavitation nuclei in the nanoscale range.

1.4.2 Therapeutic agents encapsulated in ultrasound delivery vehicles

Therapeutic ultrasound has been demonstrated to significantly improve the uptake of anti-cancer molecules of various sizes and molecular masses (e.g. plasmid DNA [158], 6-nm titanium oxide nanoparticles [168], 72 kDa clotting protein thrombin [161], 150 MDa 120-nm adenovirus [159]) in cells and tissues. According to the official U.S. Food and Drug Administration (FDA), there are presently 141 FDA-approved anti-cancer drugs. However, the majority of the ultrasound-enhanced drug delivery studies have focused on a few selected therapeutic agents. The following section reviews some of the drugs and therapeutic

nucleotides (genes and siRNA) that have been more frequently used in combination with ultrasound.

1.4.3 Conventional chemotherapies and small molecules

Doxorubicin is one of the drugs most commonly tested for ultrasound-enhanced delivery. This anthracycline has been entrapped within liposomes, nanobubbles, and microbubbles [130, 160, 162, 174, 193]. Another chemotherapeutic that has been tested in combination with ultrasound is the mitotic inhibitor paclitaxel [194-196]. In addition, ultrasound has been applied to enhance the diffusion of radionuclides to pancreatic tumour cells *in vitro* [166] and to inhibit the growth of leukaemia cells alongside the delivery of chemotherapeutic drug cytosine arabinoside *in vitro* [197]. Application of focused ultrasound in conjunction with microbubbles has been shown to enable non-invasive delivery of epirubicin across the blood-brain barrier in mouse models [198].

In addition to conventional chemotherapeutics, biological therapeutics (e.g. peptides and antibodies) have also recently been used in combination with ultrasound. Thrombin has been used as a ‘natural clotting drug’ in several delivery studies [199]. Klivanov *et al.* showed that the release of thrombin from liposomes conjugated to the surface of microbubbles was triggered by a 1 MHz ultrasound treatment at 5 pulses using a 100,000 cycle pulse length, resulting in significant acceleration of *in vitro* blood clotting [161]. Lee *et al.* also demonstrated similar thrombin-release effect using 3.5 MHz ultrasound (4.7 MPa peak rarefactional pressure) applied to thrombin-loaded microbubbles *in vitro* [200].

1.4.4 Nucleotide-based therapeutics

Serving as the basic units of nucleic acids like DNA and RNA, nucleotides offer powerful therapeutic benefit due to their intrinsic ‘amplification’ potential, with each successfully delivered copy providing many therapeutic events. Unfortunately nucleotide-

based therapeutics are often restricted *in vivo* by their relatively large extended structure and size and high net negative charge. These characteristics can inhibit extravasation from the circulation into the tumour, limit movement through the tumour interstitium and restrict passage across the plasma membrane of cells. Technologies, such as ultrasound, that can help remove these limitations therefore hold considerable potential therapeutic benefit. Ultrasound has been used in conjunction with nucleotide sequences which will instigate a therapeutic effect, such as the suppression of tumour growth, or act as a marker molecule (e.g. GFP and luciferase), to allow quantification of the efficiency of delivery. Current techniques in genetic engineering have even enabled the synthesis of hybrid therapeutic and reporter genes [201]. Therapeutic genes can be delivered using viral or non-viral vectors; while viral gene delivery will be discussed in section 1.4.5 of this thesis, this section focuses mainly on non-viral ultrasound-enhanced gene delivery.

Taniyama *et al.* reported an *in vivo* study performed in a rabbit model testing the delivery of a non-encapsulated luciferase reporter plasmid to skeletal muscle cells in the presence and absence of Optison microbubbles and ultrasound (1 MHz and 2.5 W/cm²) for 30 sec. Compared to administration of the plasmid alone the application of plasmid and ultrasound gave a 10-fold enhancement of reporter gene expression, perhaps suggesting the instigation of a sonoporation event. Notably this enhancement was increased to 70-fold when the cavitation nuclei Optison was also included. Even though this model did not utilise tumour cells, there is a strong implication that the same ultrasound-enhanced delivery mechanisms can be applied to the treatment of cancer [158]. However such a strategy may ultimately be limited to situations where direct administration into target tissue is possible, as non-encapsulated plasmid would be rapidly eliminated following intravenous administration. Suzuki *et al.* transfected S-180 cancer cells with liposomal bubbles containing plasmid DNA encoding the luciferase gene. When bubbles with plasmid were used in combination with ultrasound the magnitude of luciferase expression was two orders of magnitude higher than in

the three controls: plasmid-only transfection, ultrasound-guided plasmid transfection in the absence of liposomal bubbles, and plasmid transfection with liposomal bubbles with no ultrasound exposure [189]. The liposomes used in these studies measured 1 μm and so have an average size that is still likely to exclude them from the tumour interstitium if administered intravenously. Many other studies performed using luciferase [152, 159, 186] and GFP [159, 202] reporter genes or calcein [161, 203] have demonstrated that ultrasound alone may have a direct effect on gene expression even without the introduction of microbubbles. However, a greater level of delivery was achieved when ultrasound was applied with cavitation nuclei in all these studies, compared to the absence of ultrasound and/or cavitation nuclei.

The use of ultrasound to enhance delivery of therapeutic nucleotide such as siRNA [203-207] and suicide genes [149, 157, 208] has been the focus of much recent research. Vandenbroucke *et al.* [205] and Otani *et al.* [204] were able to conjugate siRNA to the surface of microbubbles and instigated the cavitation nuclei at 1 MHz (10% duty cycle, 2 W/cm², 2 sec) and 1 MHz (20% duty cycle, 2 W/cm², 10 sec), respectively. In the presence of ultrasound, the siRNA-loaded microbubbles were able to silence more luciferase expression (90%) than the free siRNA control (10%) in HUH7 cells [205]. Otani *et al.* further demonstrated that the siRNA-loaded microbubbles were able to knock down around 2-fold more PTEN (a tumour suppressor gene) than control siRNA alone in both adipose stromal cells and mesenchymal stem cells [204]. Negishi *et al.* [207] and Endo-Takahashi *et al.* [206] successfully formulated siRNA within liposomal bubbles for ultrasound-enhanced delivery; in contrast to previously discussed studies, in both these studies the siRNA was entrapped inside the liposomal bubbles, potentially improving *in vivo* applicability. Furthermore, no additional ultrasound contrast agents were present, and so siRNA entrapped liposomes served as the sole cavitation nuclei at 2MHz (50% duty cycle, 2.0 W/cm², 10 sec) for both experiments; again statistically significant knockdown of desired genes in tumour cells were observed [206, 207]. It is notable that all these formulations measure in the micron size range and that all these

studies have been performed *in vitro* and so the challenge will now be to test these technologies in *in vivo* studies.

Of the therapeutic genes tested, herpes simplex thymidine kinase (HSVtk) has been a popular choice in studies of ultrasound-enhanced delivery of genes to cancer cells [157, 208]. As a suicide gene, HSVtk imparts sensitivity to ganciclovir (GCV) upon cancer cells by encoding a protein that metabolizes non-toxic GCV into a phosphorylated product. This produces a toxic nucleoside analogue that induces apoptosis by inhibiting the function of DNA polymerases [209]. Aoi *et al.* demonstrated that HSVtk plasmid transfection efficiency in both *in vitro* and *in vivo* experiments was greatly increased in the presence of Optison microbubbles and ultrasound (1 MHz, 1.3 W/cm², 50% duty cycle, 10 sec). *In vitro* experiments showed that the relative number of A549 cells that underwent apoptosis in the presence of Optison, ultrasound, and HSVtk gene was around 20 times higher than following exposure to just Optison and ultrasound. However, the experiment did not address how this enhanced gene delivery compared to the apoptotic effect of transfection of the HSVtk gene only or of exposure to ultrasound alone. *In vivo* results indicated that tumour size was reduced by a factor of four after 30 days in the ultrasound-enhanced gene therapy group compared to gene therapy alone. Aoi *et al.* suggested that sonoporation (discussed earlier in section 1.3.1) is the main driving force behind improvements to suicide gene delivery, and demonstrated that exposure to the same ultrasound parameters alone did not induce any cell damage [157].

Carson *et al.* recently reported the synthesis of microbubbles (1.9-2.3 µm) with HSVtk plasmid attached to the surface and demonstrated that such attachment provided efficient protection from DNase digestion. Following intravenous injection of this formulation into mice bearing C3H/NeJ carcinoma cells, a 1.3MHz ultrasound at 1.8MPa was applied to the tumour region. Notably, the interval between each ultrasound pulse was adjusted to allow enough time for the microbubble to reperfuse in the tumour site. Ultrasound-targeted microbubble delivery was shown to increase expression of the payload gene (either GFP or

HSVtk) and retard tumour growth [208]. Interestingly expression was localised mainly in endothelial structures again emphasising the important potential that these micron sized systems may have in application to anti-angiogenic strategies.

Further studies with suicide genes have been performed by Ogawa *et al.* who introduced the recombinant fcy::fur gene, and exposed LNCap cells to this plasmid *in vitro* in combination with 1 MHz ultrasound at 0.5 W/cm² and 10% duty cycle for 60 s in the absence of any UCAs. The fcy::fur gene expression was enhanced 15-fold in the sonicated sample compared to control with no ultrasound [149]. As no UCAs were introduced in this study, it is unclear whether this effect is the result of the presence of gas bubbles in the media which may have served as cavitation nuclei or whether the ultrasound mediated a direct effect on the cell membranes.

Most of the ultrasound-enhanced gene and drug delivery studies reviewed here rely on sonoporation and do not distinguish the effects of sonoporation on cancer cells of therapeutic agents. These experiments are mainly performed *in vitro* [157, 161, 200, 205-207], and direct intratumoural administration was used in the case of most *in vivo* studies with little consideration or testing of the circulation half-life of these agents [154, 187]. The few studies that have used intravenous administration have relied on the use of microbubbles, which possess size limitations that may make them most effective when utilised in anti-angiogenic strategies rather than when used for direct tumour cell kill [155, 157, 158].

1.4.5 Ultrasound-enhanced tumour-targeting virotherapy

Clinical trials using oncolytic virotherapy for the treatment of tumours were performed as long ago as 1956 [210]. Since then, this field of research has generated many useful vectors and some impressive clinical results. However, the use of viruses in combination with ultrasound was not reported until 2006 [211]. Adenoviral vectors encoding the GFP reporter

gene (Ad5-GFP) were reconstituted in the absence or presence of microbubbles, and a 30-min at 37°C incubation with 60 mg/mL human complement was used to inactivate free non-incorporated Ad5-GFP as shown by reduced infection of a cancer cell line monolayer. The Ad5-GFP-microbubble complexes were administered to DU-145 and H23 cells plated on glass coverslips, which were then exposed to 2.5 MHz ultrasound at 535 kPa continuously for 1 min. Strong fluorescence signal was observed when the Ad5-GFP-microbubble formulation was used in combination with the ultrasound exposure. However, it remains unclear how the levels achieved differed from those resulting from the simple addition of the same amount of non-modified Ad5-GFP [211].

In further studies, Greco *et al.* showed that intravenous administration of oncolytic Ad5-GFP-microbubble complexes gave greatly enhanced GFP expression in an ultrasound exposed (2.25 MHz ultrasound 1.8 MPa continuously for 10 min) tumour compared to a non-ultrasound exposed tumour in the same mouse. This important study represents the first demonstration of such an effect and to develop this work the authors then tested two therapeutic adenoviruses for efficacy in two xenograft models. Intriguingly, regardless of the virus or model used no significant decrease in the growth of the ultrasound exposed tumour with respect to the non-exposed tumour was observed. The authors postulate that such an effect was the result of the immune ‘bystander’ effects from the successfully infected ultrasound exposed tumour providing systemic inhibition of the growth of the non-ultrasound treated tumour. Unfortunately the absence of a control group of Ad5-microbubble treated mice in which neither tumour received ultrasound prevented this hypothesis being fully validated. Hence, whilst this study represents substantial progress in testing the application of ultrasound for viral delivery, it is unclear whether ultrasound only provided enhancement of the release of the virus from the microbubbles or whether the amount of virus delivered and / or the intratumoural distribution of these viruses was also improved. A better understanding

and quantification of the mechanisms responsible (inertial cavitation, stable cavitation) will also be an important step in developing this work [212].

Instead of using conventional phospholipid microbubbles as UCAs, Mannel *et al.* introduced magnetic microbubbles (MMB) into their ultrasound-enhanced virotherapy strategy. The advantage of using MMBs is that an external magnetic field can be applied to concentrate these magnetic UCAs within the tumour, [213]. Mannel *et al.* prepared rrl-CMV-eGFP lentiviral particles and iron oxide MMB by mixing at a ratio of 300 femtogram of iron per viral particle. Human dermal microvascular endothelial cells (HMEC) were cultured and placed under a neodymium iron boron magnet for the induction of a magnetic field. All experiments were carried out at 5 lentiviral particles per HMEC and 1 MHz ultrasound (2 W/cm², 50% duty cycle, and 30 sec) was applied to the media. Measurement of viral transduction was carried out 72 hr later using flow cytometry as shown in **Figure 1-11**, and in contrast to many other studies reviewed here, care was taken to characterise the influence of each treatment component in isolation as well as in combination [213]. The combined use of virus, MMB and ultrasound provided substantial and significant improvements in infection efficacy compared to all other combinations tested. This method has therefore provided promising results, and if the use of MMB and magnetic fields can be translated for *in vivo* use it may have important clinical applications [213].

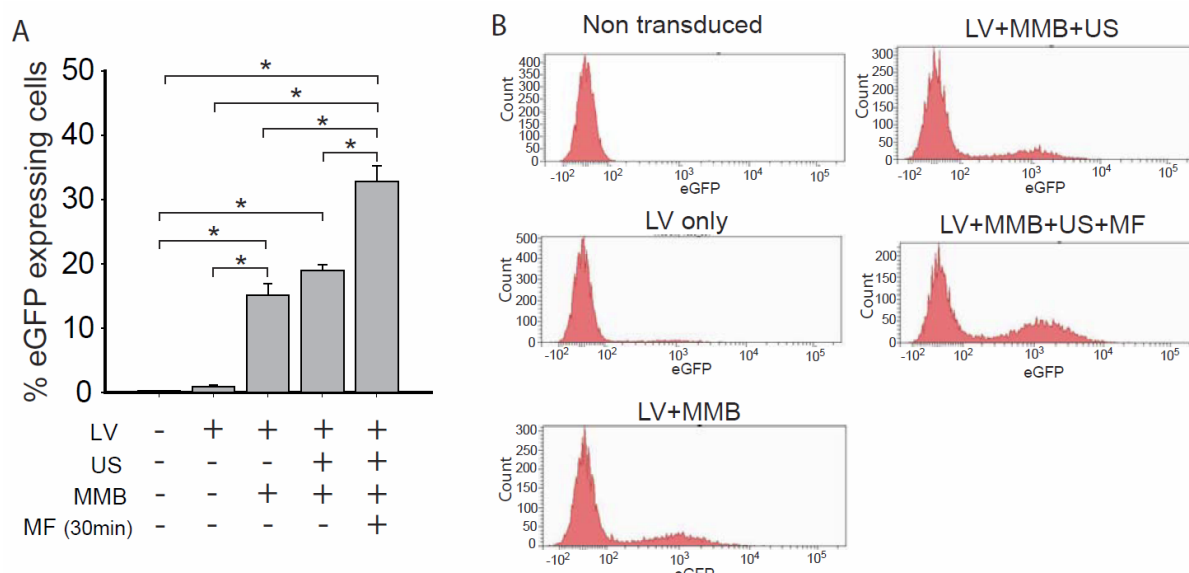


Figure 1-11: Lentiviral particle (LV) infection of HMEC in the absence and presence of ultrasound (US), magnetic microbubbles (MMB), and/or magnetic field (MF). The percentage of GFP expressing cells and distribution of GFP intensity of the same cells were shown in parts A and B, respectively. Statistical significance with $p < 0.05$ was shown with *; reproduced from [213] with permission of *Pharmaceutical Research*.

Virotherapy is an important emerging therapeutic modality, especially when direct intratumoural injection of these self-amplifying tumour cell killing agents can be achieved [214]. However the use of virotherapy for the systemic treatment of metastatic disease has so far been limited by poor extravasation into tumours and inefficient intratumoural spread. It now seems that therapeutic ultrasound may offer a solution to this limitation and the combination of these two relatively new and powerful technologies offers much promise. A glut of recent *in vitro* studies have clearly demonstrated ultrasound can provide a powerful stimulus in this context and the test will now be whether these findings transfer to *in vivo* studies. It is also clear however that understanding, quantifying and optimising the underlying mechanisms by which ultrasound instigates enhancement of virotherapy will be essential in achieving this goal.

1.5 Therapy safety

1.5.1 Virotherapy

The current understanding and control of Ad5 triggered immune response and tropism to various organs is very mature and enables researchers to develop better and safer virotherapies [215]. Clinical trials using oncolytic virotherapy for the treatment of tumours were performed as long ago as 1956 [210]. Since then, this field of research has generated many useful vectors and some impressive clinical results.

The most well-known Ad5 therapy is probably ONYX-015, which lacks E1B gene. Without this gene, Ad5 is unable to replicate in cells with a working p53 pathway, which is deficient in most tumour cells due to mutations; hence, several studies have shown that ONYX-015 could replicate in and lyse the host cancer cells but not healthy cancers [216]. Having already passed the phase I clinical trials, ONYX-015 is now being tested for the treatment of a wide range of p53-deficient cancers in phase II clinical trials [215]. In these human clinical trials, ONYX-015 has been comprehensively tested for treatment of various forms of cancers: brain, colorectal, hepatic, pancreatic, prostate, and ovarian; all these trials not only show significant improvement but also demonstrates recipients of ONYX-15 were well tolerated [217].

Several versions of adenoviruses have been approved by the FDA and are currently being tested for phases III of clinical trial [218, 219]. Recent human trials of oncolytic adenoviruses have shown consistent safety [215]. In fact, the Chinese State Food and Drug Administration already approved the world's first oncolytic virotherapy treatment with combination with chemotherapy to treat patients with nasopharyngeal cancer [220].

Besides Ad5, other forms of oncolytic viruses have shown great promises in clinical trials, including reovirus and herpes simplex virus. After being intravenously administrated to

terminal-stage cancer patients with metastatic melanoma in a phase II clinical trial, reovirus type 3 Dearing (RT3D) showed low toxicity and improved the median survival rate of these terminal-stage cancer patients from 45 days to 165 days [221]. As a result, RT3D has now been approved by FDA for phase III clinical trial, and the study is ongoing [222]. In addition, OncoVEX GM-CSF is probably the most promising oncolytic herpes simplex virus. BioVex Group (the original patent holder of OncoVEX) was purchased by Amgen at the price of \$1 billion USD in 2011 [223]. In March 2013, Amgen announced its early OxonVEX GM-CSF phase III results: after intravenous administration of OncoVEX GM-CSF, 24% of the stage III and IV melanoma cancer patients showed complete regressions of tumours, compared to 0% from the un-treated group; this is statistically significant with $p < 0.001$ [224, 225].

These encouraging news reinforce the idea that rigorously tested and safely designed oncolytic virotherapy could offer many windows for safe cancer therapy in the future. However, most of the clinically approved oncolytic viruses have been extensively genetically engineered. Although genetic engineering can be used to modify Ad5 capsid proteins to avoid non-target cell infection and interaction with pre-existing immunity [99], the magnitude and complexity of the changes needed often leads to inefficiencies in viral production and eventual loss of infective capacity [100]. Offering extended plasma circulation, avoidance of interaction with human erythrocytes, and decreased levels of hepatocyte toxicity in animal model, PEGylated or PHPMA-coated Ad5 has yet to gain approval to be tested in clinical trial. A combination of genetically engineered oncolytic viruses with an extra surface coating (e.g. to prevent interacting with blood proteins and avoid being detected by immune system) will be a promising and safe route for future treatment of cancer.

1.5.2 Therapeutic ultrasound

Ultrasound therapy offers a safe, convenient, non-invasive, and tissue-specific system to deliver drugs and genes to solid tumours [155, 157, 189]. A crucial prerequisite for any cancer therapy is that the benefits of killing cancer cells outweigh deleterious side effects and damage to healthy cells. All studies discussed in this chapter used 0.5 to 2.5 MHz ultrasound transducers with up to 2.0 MPa pressure and showed no significant difference in cell viability in the absence or presence of therapeutic ultrasound alone [159]. Although inertial cavitation and sonoporation might create short-term pores in cell membranes for delivery of therapeutic agents, these pores quickly close upon the removal of ultrasound exposure [127, 144]. The majority of sonoporation studies showed no decrease in cell viability with ultrasound alone, demonstrating that increases in cancer cell death were instigated by the anti-cancer therapeutics [149, 150].

Furthermore, in theory, because ultrasound is a non-invasive wave with acoustic energy concentrated at the focus (e.g. within the solid tumour), all tissues and organs in the path of the ultrasound wave but outside the focal region should not suffer ultrasound-induced damage.

The concern that ultrasound-induced inertial cavitation may create shear forces that induce the breakup of primary cancer cells thereby enhancing metastasis was recently assuaged. Oosterhof *et al.* showed that there is no difference in the observed metastases between control and ultrasound-treated xenograft mice [226]. Whilst Wu *et al.* studied the effect of therapeutic ultrasound on human patients and concluded that it does not increase the chance of metastasis [227]. However, this is not conclusive, for more experiments need to be carried out to verify the claim.

1.6 Implications for the present work

The mainstream non-invasive therapies used for treating solid tumours are chemotherapy and radiotherapy. These methods are limited by sub-optimal specificity for cancer rather than normal cells and the possibility that they induce suppression of the host anti-cancer immunity. Therapeutic ultrasound is a non-invasive modality which does not impair immune function and is not associated with any common physiological insult. As such it represents an ideal technology for use in enhancing the specificity and efficacy of mainstream therapies. On the other hand, ultrasound therapy is not a cure-all method either and does have its limitations. Sound waves propagate poorly in air and through bone; therefore, therapeutic ultrasound is not appropriate to target tumours in or close to lung or bowel tissue and is difficult to apply through the skull. If a sufficient acoustic window is not present or if the targeted tissue or organ is situated too close to other healthy tissues, it is not possible to treat these tumours with therapeutic ultrasound.

The integration of virotherapy with therapeutic ultrasound is an encouraging field worthy of further investigation, not least because the self-amplification of the virus can overcome issues associated with delivering sufficient therapeutic dose. Cancer cell-specific viruses can be genetically engineered to provide an enhanced specificity for tumours while avoiding viral infection to other healthy tissues. Given the many therapeutic systems already in clinical use [127, 228] and the approval by the FDA for several adenoviruses to be used in Phase III clinical trials [218, 219], ultrasound-enhanced virotherapy may well be rapidly approaching clinical applicability.

In summary, this thesis focuses on the further development of ultrasound-enhanced virotherapy; in particular, a successful anti-cancer therapy merging these two promising technologies should:

- 1) Utilise the benefit of the EPR effect in leaky tumour vasculature by
 - i. Controlling the size of Ad5 therapeutics to be less than 200 nm.
 - ii. Avoiding the interaction with erythrocytes or other blood proteins.
- 2) Extend the plasma circulation of Ad5 by
 - i. Coating Ad5 surface to avoid interaction with blood components such as antibodies.
 - ii. Conjugating Ad5 with other nanoparticles with long plasma half-life.
- 3) Lower the liver capture of Ad5 by
 - i. Ensuring Ad5 hexon, penton, and fibre are well protected by coating.
 - ii. Using genetically engineered Ad5 that has reduced liver infection.
- 4) Accumulate Ad5 at significantly higher levels in target tumours by
 - i. Applying ultrasound with cavitation nuclei to increase Ad5 extravasation.
 - ii. Improving the plasma circulation and EPR effect, as described above.
- 5) Present a controllable trigger to de-stealth Ad5 only in tumour environment by
 - i. Achieving effective coating whilst only minimally modifying Ad5 surface.
 - ii. Introduce disulphide bonds that link between Ad5 and the coating.
- 6) Test if the newly proposed therapy is more effective than existing alternatives by
 - i. Comparing results with Ad5–PEG.
 - ii. Comparing results with Ad5–PHPMA.
- 7) Optimise the inertial cavitation energy delivered to the tumour by
 - i. Refining the optimal ultrasound parameters.
 - ii. Delivering the ultrasound beam at various regions of the same tumour.
 - iii. Minimising the harm and pain received by the animal models.

CHAPTER 2

DESIGN OF A NEW ADENOVIRUS STEALTHING STRATEGY

2. Design of a new adenovirus stealthing strategy

2.1 Challenge of adenovirus therapy

As reviewed in section 1.2, several limitations have prevented the intravenous (i.v.) delivery of adenovirus (Ad5) for the treatment of metastatic cancer, i.e. the disease in its most common and fatal form [67, 86]. Notably rapid blood cell and reticuloendothelial system mediated clearance, poor target cell selectivity, and instantaneous antibody binding lead to efficient sequestration and neutralisation of i.v. delivered Ad5 before it can reach target tumours [87]. To increase the potency of systemically administered Ad5, its circulation half-life must be increased and binding to non-target cells avoided [88]. Several Ad5 capsid proteins are responsible for both its cellular tropism and *in vivo* sequestration: with RGD sequences in the penton base [89], hypervariable regions of hexon, and knob and shaft domains of the fibre playing a role in the Ad5 infection pathway [68] and also being common targets for pre-existing immunity [90].

Although genetic engineering can be used to modify these Ad5 capsid proteins to avoid non-target cell infection and interaction with pre-existing immunity [99], the magnitude and complexity of the changes needed often leads to inefficiencies in viral production and eventual loss of infective capacity [100]. Chemical modification of therapeutic agents can improve their bloodstream compatibility and ultimately their accumulation at target sites of disease [101, 229, 230]. Such chemical ‘stealthing’ has been particularly useful in modifying Ad5, a powerful and selective anti-cancer agent. Indeed, the addition of amine-reactive hydrophilic polymers, such as polyethyleneglycol (PEG) or poly-[*N*-(2-hydroxypropyl)methacrylamide] (PHPMA), provides a simpler, more effective and non-heritable method of modifying Ad5 than genetic engineering [100, 103]. Stealthing Ad5 with PEG or PHPMA can dramatically reduce liver capture and enhance bloodstream circulation and tumour accumulation in murine pre-clinical models [105]. However, stealthing may still

not provide sufficient coating of Ad5 to allow effective intravenous administration in humans. In particular, conventional polymers provide little protection of the acidic regions of the Ad5 capsid. To improve stealthing of these regions quaternary amine groups have been incorporated into PHPMA, but this inclusion can result in such a dense coating that Ad5 infection activity cannot be fully recovered [106].

The aims of the work described in this chapter were to:

- 1) Propose a new Ad5 shielding solution that combines the advantages of both PEG and PHPMA to address current challenges in virotherapy using chemical modification.
- 2) Confirm that the proposed new structure ('Ad5–AuPEG') would be synthesisable and could maintain good structural integrity as assessed by dynamic light scattering, zeta potential, protein gel separation, and transmission electron microscopy.
- 3) Discuss the decisions and methods used to create tumour-like reducing conditions and test the reducibility of Ad5–AuPEG to detach AuPEG.
- 4) Compare Ad5–AuPEG with previously published Ad5–PEG and Ad5–PHPMA in terms of size, surface charge, and density.
- 5) Quantify the stoichiometry of PEG attached per Au and AuPEG attached per Ad5.

2.2 Newly proposed platform to shield Ad5

The development of a novel platform stealthing chemistry and its application to the model nano-medicine Ad5 is reported here. Gold nanoparticles (Au) bearing multiple 2kDa PEG molecules were attached to Ad5 via a single reduction-cleavable 5kDa PEG, to give Ad5–AuPEG (**Figure 2.1**). There are two proposed benefits of this design. First, it was hypothesized this 'dandelion'-like structure would provide a dense protective steric shield similar to that achieved with multivalent PHPMA-based polymers whilst still relying on only

minimal monovalent attachment of the shield to the Ad5, as with conventional PEG coating. Secondly, increasing the density of the therapeutic particles (Ad5 in this case) may improve transport due to ultrasound-induced microstreaming and shock waves thereby increasing the degree and depth of their penetration into the tumour.

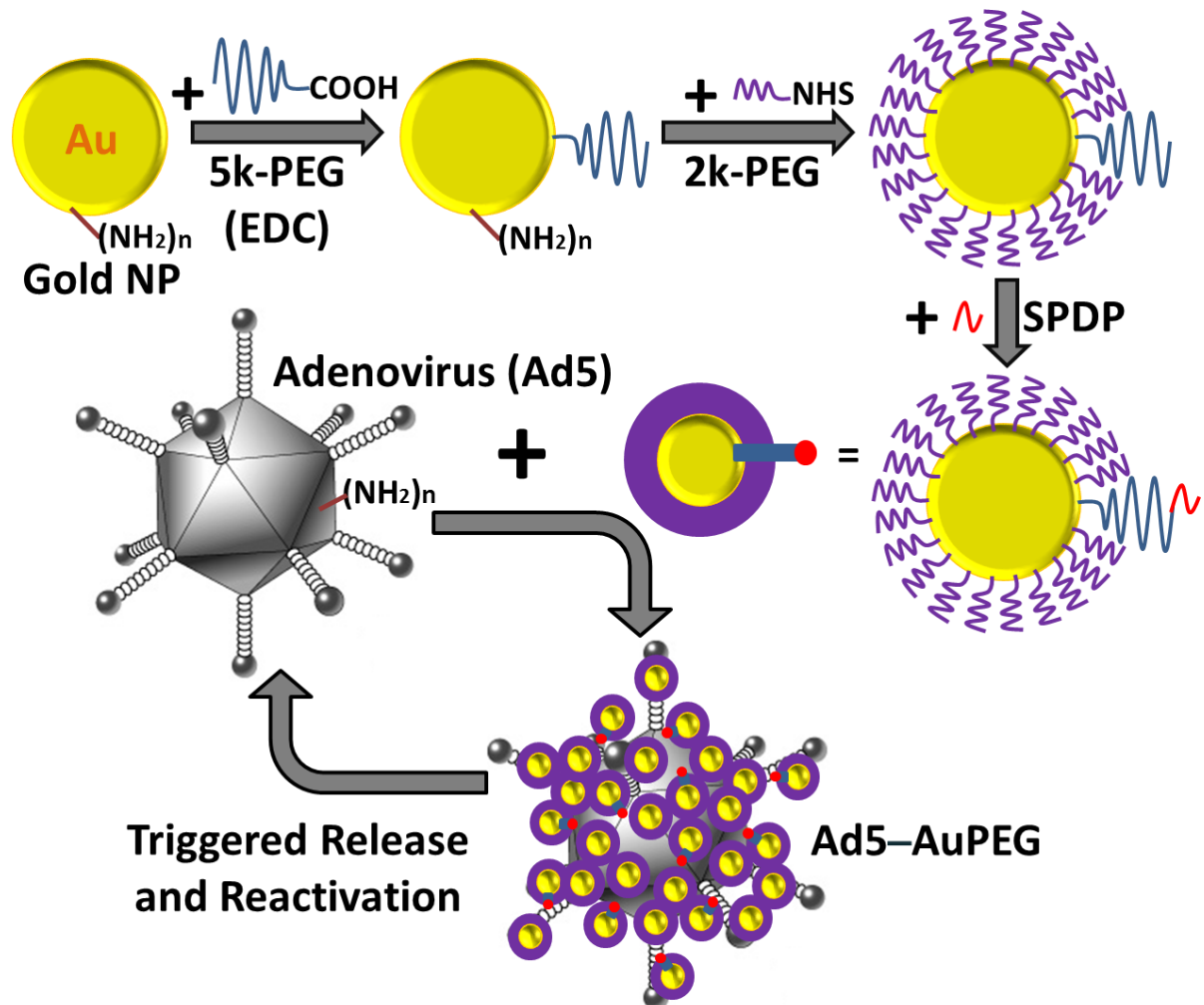


Figure 2.1: Schematic of synthesis of PEGylated gold-coated adenovirus (Ad5-AuPEG). A novel coating of adenovirus (Ad5) using cleavable PEGylated gold nanoparticles (AuPEG) with the introduction of a disulphide bond to allow reduction-triggered cleavage upon exposure to the tumour environment [231, 232]. A dense protective steric shield is provided with only minimal modification of the Ad5 capsid surface.

In addition, Au nanoparticles offer several characteristics which make them a useful component for the inclusion in nano-medicines [101]. PEGylation of gold nanoparticles [233] or nanorods [234] has been shown to extend their blood circulation half-life. PEG is a biodegradable polymer that provides good steric hindrance and protection at atomic level by preventing bloodstream proteins and antibodies from binding to the delivered nano-medicine.

Overlapping electron clouds formed by the atoms in each PEG molecule further contribute to the overall shielding [235]. In fact, studies have shown that whereas Au is effectively cleared from the bloodstream of mice within 1 hr, AuPEG exhibits much longer circulation half-life, e.g. 12 hr for 5k-PEGylated 20-nm Au [236], 16 hr for 5k-PEGylated 4-nm Au [237], and 28 hr for 5k-PEGylated 13-nm Au [238]. By conjugating AuPEG to Ad5, the steric protection and density of the Ad5 was increased with the ultimate aim of improving *in vivo* circulation half-life and response to ultrasound.

While Chapters 2 and 3 focus on the development and testing of Ad5–AuPEG to characterise the level of stealthing provided, Chapters 4 and 5 present investigations into the influence of AuPEG addition on the extravasation of Ad5 into and through tumours in response to ultrasound exposure.

In summary, the ‘dandelion’ structure described and tested here was designed to enhance steric shielding and response to ultrasound thereby potentially broadening the clinical utility of this and other powerful therapeutic agents by enabling their successful intravenous injection for the treatment of metastatic cancer.

2.3 Synthesis and reactivation of Ad5–AuPEG

A schematic summary of the synthesis of dandelion-like structure Ad5–AuPEG is shown in **Figure 2.1**. All reactions described below were performed in 1.5-mL Lo-bind tubes (Eppendorf, Germany). A sample of 1×10^{14} spherical amine-presenting 5-nm Au (~300 amine groups per Au particle, MW 1.96 MDa; Nanopartz, USA) was first conjugated to 5kDa carboxyl-PEG-thiol (Rapp Polymere, Germany) via EDC cross-linker chemistry in 1mL PBS (pH 4.5) at molar ratio of 1 Au : 10 5kDa-PEG : 50 EDC for 1 hr at 25 °C. Amicon ultra-4 centrifugal filters (AUCF; Millipore, UK) with molecular weight cut-off of 100kDa were used to filter out excess 5kDa-PEG. 5kDa-thiol-PEG–Au was later conjugated to 2kDa methyl ether-PEG-*N*-hydroxysuccinimide (Rapp Polymere, Germany) in 1-mL PBS (pH 7.4) at molar

ratio of 1 x 5kDa-PEG–Au : 2000 x 2kDa-PEG for 2 hr at 25 °C. AUCF was again used to filter out excess 2k-PEG. Gold nanoparticles fully saturated with the 2kDa-PEG-methyl ether and 5kDa-PEG-thiol (AuPEG) were reduced in 1M DTT buffer (pH 8) for 30 min to break any possible disulfide bonds formed, and purification was performed by AUCF. AuPEG was reacted with heterobifunctional reagent SPDP in 1mL PBS (pH 7.4) at molar ratio of 1 AuPEG : 5000 SPDP for 1 hr at 25 °C and purified by AUCF. The two functional groups, N-hydroxysuccinimide ester (NHS) and 2-pyridyldithio, on SPDP can react with amine and thiol groups, respectively, and are widely used in many other chemistry conjugations [239].

The final conjugation step was to react AuPEG–SPDP with Ad5-GFP (The Native Antigen Company, UK) in PBS (pH 7.4) at molar ratio of 1 Ad5 : 2000 AuPEG–SPDP for 1 hr at 25 °C. There are 1,800 free amine groups on each Ad5 particle for potential reaction [104]. Final Ad5–AuPEG particles were further purified by gel filtration on a Sephacryl S-500 high resolution gel filtration media (GE Healthcare Life Sciences, USA) column equilibrated with PBS (pH 7.4) and re-concentrated to $> 1 \times 10^8$ viral particles/ μ L in PBS with 10% glycerol (pH 7.4) for storage at 4 °C. The Sephacryl S-500 gel volume was 35mL (the column's circular base diameter was 1.5 cm and height was 20 cm). PicoGreen assay [240] was used to quantify the final Ad5–AuPEG viral particle concentration. After these conjugation and purification steps, the percentage yield for Ad5–AuPEG was between 30% and 50% (data not shown). The efficiency of PEGylation of Au and conjugation of Ad5–AuPEG was monitored by assaying the reduction in free amine groups using the TNBS assay, as described later in section 2.6.5.

The disulphide bond between the Ad5 and the AuPEG provided a mechanism for triggered de-stealth and infection reactivation designed to be responsive to the reducing conditions within tumors [231, 232]. To test if triggered release of the AuPEG could be achieved, Ad5–AuPEG was incubated in PBS containing 10 mM β -mercaptoethanol (BME; Sigma-Aldrich, UK) for 20 min. 10 mM BME was chosen as the threshold level of reducing

buffer because it has a reduction potential of -260 mV [241], which matches the reduction potentials of many reported tumour micro-environments [232, 242]. In addition, the pH value of PBS with 10 mM BME was measured 6.92 ± 0.08 ($n = 5$), which corresponds to other reported tumour interstitial pH range 6.9 to 7.1 (healthy tissues have a typical pH range of 7.2 to 7.4) [243]. AUCF and gel filtration were used to purify excess BME and AuPEG by exchanging the reaction buffer with PBS (pH 7.4). Unless otherwise notice, all data labelled '+ BME' shown in this thesis represents incubation with 10 mM BME for 20 min. Incubation of Ad5 with 10 mM BME for over 30 min was shown to decrease un-modified control Ad5 infectivity by more than 20% (data not shown); as a result, the 20 min time point was chosen as a standard throughout the rest of these reduction response experiments.

2.4 Syntheses of Ad5-PEG and Ad5-PHPMA

Besides having a negative control (un-modified Ad5) to compare to for further experiments, two positive controls/comparators were also synthesised because of their widespread previous use in the stealthing of Ad5 and other therapeutics: Ad5-PEG and Ad5-PHPMA [100, 103].

The synthesis of Ad5-PEG was carrying out by adding 25 μL of the 20 mg/mL of amine-reactive NHS-20kPEG (dissolved in DMSO; Rapp Polymere, Germany) and 61 μL of Ad5-GFP (1.63×10^9 Ad5 per μL ; the Native Antigen Company, UK) into a 1.5-mL Lo-bind tube containing 914 μL of HEPES buffer solution (pH 7.4). The molar reaction ratio of Ad5 to 20kPEG was 1 to 300,000. The reaction proceeded for 2 hr at 25°C before excesses 20kPEG were filtered out by AUCF. This simple NHS-amine chemistry was optimal in reaction buffer between pH 7 and 8.

An amine-reactive PHPMA, poly-[*N*-(2-hydroxypropyl)methacrylamide], in solid powder form was a gift from by Dr. Richard Laga (Department of Oncology, University of Oxford). The characterisation of PHPMA (carried out by Dr. Laga) showed that it contained

6.2 molar percentages of thiazolidine-2-thione (TT) amine-reactive groups in side chains and had a molecular weight of 37,000 g/mol; this translates to roughly 14 TT groups per PHPMA polymer. The synthesis of Ad5-PHPMA was carried out by adding 500 μL of 40 mg/mL PHPMA-TT (dissolved in HEPES buffer) and 61 μL of Ad5-GFP (1.63×10^9 Ad5 per μL) into a 1.5-mL Lobind tube containing 439 μL of HEPES buffer solution (pH 7.4). The molar reaction ratio of Ad5 to PHPMA is 1 to 3.2×10^6 . The reaction proceeded for 2 hr at 25°C before excess PHPMA were filtered out by AUCF. The TT groups react with primary amines on the Ad5 capsid surface under these conditions.

Since both PEG and PHPMA were covalently linked to the surface of Ad5 without any reducible functional group, the reactivation of Ad5-PEG or Ad5-PHPMA for triggered release is not possible [106]; therefore, the original and reduced results are shown only for Ad5-AuPEG in this DPhil thesis, whereas all studies with Ad5-PEG and Ad5-PHPMA were performed in a non-reducing environment.

2.5 Quantification of Au and Ad5

Throughout the modification procedures of Au and Ad5, several purification and re-concentration steps took place, making 100% yield very unlikely; as a result, a systematic way to quantify the total amount of Au and Ad5 in any reaction step was important. Fortunately, the concentration characterisations of the Au and Ad5 used in this thesis have been established by Nanopartz and in previous studies on Ad5 [240], respectively. Verification of these characterisations was performed twice to confirm the report published by Nanopartz.

All 5-nm Au particles used in this DPhil study were purchased from Nanopartz (Loveland, Colorado, USA) and supplied with product information including the molar extinction coefficient of the Au ($1.43 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$ at absorbance = 530 nm). Once the absorbance of a diluted Au sample was measured inside polystyrene standard cuvettes

(Malvern, UK) by a spectrometer, the Au concentration could therefore be determined by applying the Beer–Lambert law:

$$A = \epsilon lc$$

where A is the absorbance measured in optical density (OD), ϵ is the molar extinction coefficient measured in $\text{M}^{-1}\text{cm}^{-1}$, l is the length travelled by the light measured in cm, and c is the concentration measured in moles/L (M). Because OD is measured based on a log scale, any measurement lower than 0.1 or higher than 1.0 is considered outside the range that can be accurately measured; as a result, when making the Au sample measurement, the dilution factor was adjusted accordingly until an OD reading was obtained between 0.1 and 1.0. In addition, since the length of the cuvettes used for the measurement was 1 cm, the ' l ' term can be cancelled out directly, making the concentration $c = A/\epsilon$. Finally, the concentration c was multiplied by the dilution factor to obtain the actual Au concentration of the original sample. The actual concentration (M) was multiplied by the volume (L) and then by the Avogadro constant (6.022×10^{23}) to obtain the total number of Au particles in the sample.

To quantify sample Ad5 viral concentration, a PicoGreen assay was performed. First, 1x TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer and 1x TE buffer with 0.05% SDS (sodium dodecyl sulphate) were made. 2 μL of the purified Ad5 modifications (Ad5–PEG, Ad5–PHPMA, and Ad5–AuPEG) was mixed with 198 μL of 1x-TE-SDS in a 0.5-mL Eppendorf tube, which was then placed inside a thermo block holder for 30 minutes at 56°C. Concentrated stock lambda DNA (Sigma-Aldrich, UK) was used to make the standard curve samples by one-half serial dilution at 800 ng/mL, 400 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, and 0 ng/mL (as a negative control). 15 μL of the 200x PicoGreen dye (Fisher Scientific, UK) was mixed with 1.485 mL of 1x TE buffer to make 1.5 mL of 1x PicoGreen buffer. After the 30-min incubation, diluted Ad5 sample(s) and the DNA standard were pipetted inside a black half-full 96-well plate at 50 μL per well. The freshly

made 1x PicoGreen buffer was then pipetted into the wells to mix thoroughly with sample Ad5 and DNA standard at 50 μL per well. The plate was read at 485-nm excitation wavelength and 535-nm emission wavelength using a VICTOR² D fluorometer (PerkinElmer, USA). The fluorescence readings of the DNA standard curve were used to draw a ‘best fit’ linear regression line, which was then used to estimate the concentration (g/mL) of the sample Ad5 modifications. A good linear regression line should have a ‘coefficient of determination’ R^2 value that is greater than 0.95. The average molecular weight of a DNA molecule is 325 g/mole, and each Ad5 has 36 kb of DNA. This means the total DNA molar mass per Ad5 is $36 \text{ kb} \times 2 \text{ strands} \times 325 \text{ g/mole} = 2.34 \times 10^7 \text{ g/mole}$. Lastly, by dividing the final Ad5 DNA concentration by $2.34 \times 10^7 \text{ g/mole}$, multiplying by its volume (mL), and multiplying by the Avogadro constant, the total number of Ad5 for each sample could be determined.

In summary, after the quantification of Au modification, the percentage yield of AuPEG production from Au was around 50~80%. Based on the PicoGreen assay, the final Ad5 percentage yields of making Ad5-PEG, Ad5-PHPMA, and Ad5-AuPEG (based on the protocols described in sections 2.3 and 2.4) were around 60~80%, 50~70%, and 25~50%, respectively. In terms of the repeatability of protocols detailed in this chapter, the success rates (defined by data obtained from DLS, zeta potential, and ELISA) for making AuPEG and Ad5-AuPEG were greater than 90% and 50%, respectively. The success rates of making Ad-PEG and Ad-PHPMA were greater than 80%.

2.6 Characterisation of the newly synthesised viral therapeutics

Successful coating of Ad5 with cleavable AuPEG, non-cleavable PEG, and non-cleavable PHPMA was tested using dynamic light scattering (DLS), zeta potential, SDS-PAGE silver staining, transmission electron microscopy (TEM), and quantification of the amine groups present on Au and Ad5. All final samples were passed through 220-nm syringe filters (diameter = 33 mm) to get rid of possible reaction aggregates before size and ζ -

potential were measured. All statistical analyses in this paper were completed using a one-way analysis of variances (ANOVA) followed by a Newman-Keuls test for pairwise comparison of sub-groups. All data are typical of three or four repeat experiments. All data are reported such that *, **, and *** represent p-value < 0.05, 0.01, and 0.001, respectively.

2.6.1 Results: dynamic light scattering

To assess successful conjugations of two nanoparticles, a reliable measurement of all size changes throughout the process was needed. Zetasizer Nano ZS (Malvern, UK) was used to size all Au, Ad5, and other nano-size conjugates reported in this thesis. According to the manufacturer, the sensitivity of the DLS machine is between 0.3 and 10,000 nm, serving as a good analyser for the range of all nanoparticles used in this study. Unless otherwise noted, all samples were measured inside polystyrene standard cuvettes (Malvern, UK) which contained 1-mL PBS. Each sample was read for three trials at 25°C, and each trial contained 10 runs of reading. Therefore, the 30 readings per sample allowed the precise calculation of mean diameter and polydispersity index (PDI) of nanoparticles sized. At least 1×10^{12} particles of Au, Au-5k-PEG, and Au-5k-2k-PEG and at least 5×10^9 copies of Ad5, Ad5-PEG, Ad5-PHPMA, and Ad5-AuPEG were measured by the DLS machine. All the sizing shown here was performed directly after the various Ad5-conjugate syntheses and values obtained were shown to be stable for at least 24 hr after the syntheses.

DLS analysis of particle intensity (**Figure 2.2**) showed AuPEG (Au-5k-2kPEG) had a greater hydrodynamic diameter (15 ± 0.96 nm) than Au, which was measured as 6.3 ± 0.76 nm. Au-5kPEG was larger only by 0.1 nm compared to Au, suggesting very few copies of 5kPEGs were added to Au. When fully saturated with 2kDa PEGs, AuPEG increased to 15 nm, showing that the majority of the PEGs attached to Au were 2kDa since the Au diameter increase was much large than when only 5k-PEG was conjugated. In addition, PDI also increased from 0.229 for Au, to 0.235 for Au-5kPEG and to 0.272 for Au-5k-2kDa PEG. The

increase in PDI was only slightly. A one-way ANOVA test showed the size of AuPEG to be significantly larger than that of Au ($p < 0.001$).

Au Modification	Diameter (nm)	PDI
Au	6.3 ± 0.76	0.229
Au-5kPEG	6.4 ± 0.87	0.235
Au-5k-2kPEG (AuPEG)	15 ± 0.96	0.272

Ad Modification	Diameter (nm)	PDI
Ad	117 ± 0.9	0.075
Ad5 + AuPEG (peak 1)	18 ± 1.6	0.276
Ad5 + AuPEG (peak 2)	114 ± 1.2	
Ad5–AuPEG	149 ± 4.2	0.194
Ad5–AuPEG + BME	120 ± 1.8	0.125

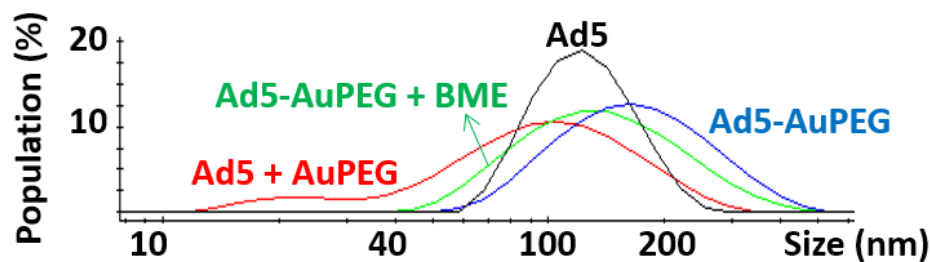


Figure 2.2: Influence of Au and Ad5 modifications on particle size and heterogeneity. Analysed by particle intensity, dynamic light scattering was used to characterise the polydispersity index (PDI) and mean diameter $\pm$ standard deviation (S.D.) of Au, Au-5kPEG, Au-5k-2kPEG (AuPEG), Ad5, Ad5 mixed with AuPEG (Ad5 + AuPEG), Ad5 conjugated to PEG (Ad5–AuPEG), and Ad5–AuPEG in the presence of reducing buffer BME ($n = 4$). One-way ANOVA analysis was performed.

Un-modified Ad5 was measured as 117 ± 0.9 nm, whereas Ad5–AuPEG was measured as 149 ± 4.2 nm, a 32-nm increase which corresponds to the combined size of two AuPEG, suggesting a good AuPEG coating surrounding Ad5. DLS analysis of identically processed non-linked Ad5 and AuPEG (Ad5 + AuPEG) distinguished two peaks with means of 18 nm for AuPEG and 114 nm for Ad5. In the ‘Ad5 + AuPEG’ sample, the decrease of ‘real’ diameter from 117 to 114 nm for Ad5 and the increase of ‘real’ diameter from 15 to 18 nm suggested that although the DLS could separate out the two populations, there was

nevertheless some interference between the two distinct particles when the light was scattering from them for DLS detection and analysis. Upon exposure to reducing environment and after filtering out the excess AuPEG, Ad5–AuPEG + BME showed a decrease in diameter back to 120 nm; while this served as a good indication that majority of the AuPEGs were now detached from Ad5, the NHS-thiol (from the reduced SPDP linker) adducts may have contributed to the slight increase in the size of reduced Ad5 (120 nm) compared to un-modified Ad5 (117 nm). These adducts also have the potential to decrease the infectivity of Ad5 (see Chapter 3 for *in vitro* cell infection data). ANOVA analysis indicated that Ad5–AuPEG was significantly greater in size than both un-modified Ad5 and reduced Ad5 ($p < 0.001$) and that there was no difference between the size of un-modified Ad5 and reduced Ad5 ($p > 0.05$). The PDI of Ad5 shifted from 0.075 to 0.194 when attached to AuPEG; upon reduction of AuPEG, the PDI decreased down to 0.125. This PDI trend indicated that the remaining NHS-thiol adduct attached to Ad5 (after reduction) made the reduced Ad5 more polydisperse than un-modified Ad5.

	Size $\pm$ S.D. (nm)	PDI
Ad5	117 $\pm$ 0.9	0.075
Ad5–PEG	151 $\pm$ 7.7	0.218
Ad5–PHPMA	146 $\pm$ 5.6	0.203
Ad5–AuPEG	149 $\pm$ 4.2	0.194

Table 2.3: Summary of size measurement of Ad5–PEG and Ad5–PHPMA and Ad5–AuPEG. Dynamic light scattering showing the polydispersity index (PDI) and mean diameter $\pm$ S.D. of Ad5, Ad5–PEG and Ad5–PHPMA, and Ad5–AuPEG ($n = 4$).

The sizing data of Ad5–PEG and Ad5–PHPMA (along with Ad5 and Ad5–AuPEG) are shown in **Table 2.3**. There was no statistical difference ($p > 0.05$) in size between Ad5–PEG (151 nm), Ad5–PHPMA (146 nm), and Ad5–AuPEG (149 nm). However, all three Ad5 modifications were significantly larger than Ad5 (117-nm) ($p < 0.001$). The reported PDI values of the three Ad5 modifications were similar (between 0.19 and 0.21), around three times larger than the PDI of Ad5 at 0.075.

2.6.2 Results: zeta potential

Virtually all nanoparticles which suspend in aqueous solutions will produce surface charge by the ionisation of their surface functional groups; zeta potential is the measurement of such surface charge and strongly mediates how nanoparticles interact with each other in solution. As a result, chemical surface modification of charged nanoparticles will result a shift in their ζ -potential. A Zetasizer Nano ZS (Malvern, UK) was used to measure ζ -potential following Au and Ad5 modifications. According to the manufacturer, the size and concentration sensitivities of Zetasizer Nano ZS are 3.8 nm to 100 μ m and 70 μ M, respectively, to obtain accurate ζ -potential measurements; all particles measured in this study were within this size range and samples had concentrations above the minimum required. Unless otherwise noted, all samples were suspended in 1-mL PBS (pH 7.4) and measured inside a 'surface zeta potential cell' (Malvern, UK). Each sample was read for three trials at 25°C, and each trial contained 50 runs of reading. Therefore, the 150 readings per sample were used to calculate the final ζ -potential of all control and modified nanoparticles and virus samples. At least 1×10^{12} copies of Au, Au-5k-PEG, and Au-5k-2k-PEG (AuPEG) and at least 5×10^9 copies of Ad5, Ad5-PEG, Ad5-PHPMA, and Ad5-AuPEG were used for each ζ -potential measurement.

Results from ζ -potential measurements (**Figure 2.4**) demonstrated that positively charged Au was coated successfully since ζ -potentials became less positive as amine groups on the Au were removed by reaction with PEG, changing from 2.6 to 1.5 mV ($p < 0.01$) upon the addition of 5kDa PEG and to 0.2 mV ($p < 0.001$) after subsequent addition of 2kDa PEG. The almost neutral ζ -potential of AuPEG indicated that most of the amine groups were saturated with PEG (no charge). The ζ -potential of Ad5 increased significantly ($p < 0.01$) from -16.9 mV to -10.8 mV upon reaction with the less negative AuPEG. The positive control, a mix of unconjugated Ad5 and AuPEG gave a ζ -potential of -18.9 mV, which was not significantly different ($p > 0.05$) to the ζ -potential of Ad5 alone. In addition, in the case of

Ad5–AuPEG + BME, the reported ζ -potential decreased to -16.1 mV and was no different ($p > 0.05$) from the ζ -potential of un-modified Ad5, showing that majority of AuPEG were successfully reduced and detached from Ad5. While there were some NHS-thiol adducts attached to the reduced Ad5, they carried no charge and did not change the overall ζ -potential of the Ad5. Added to the DLS data shown in the previous section, these results provided further evidence that the surface modifications of Au with PEG and Ad5 with AuPEG were successful.

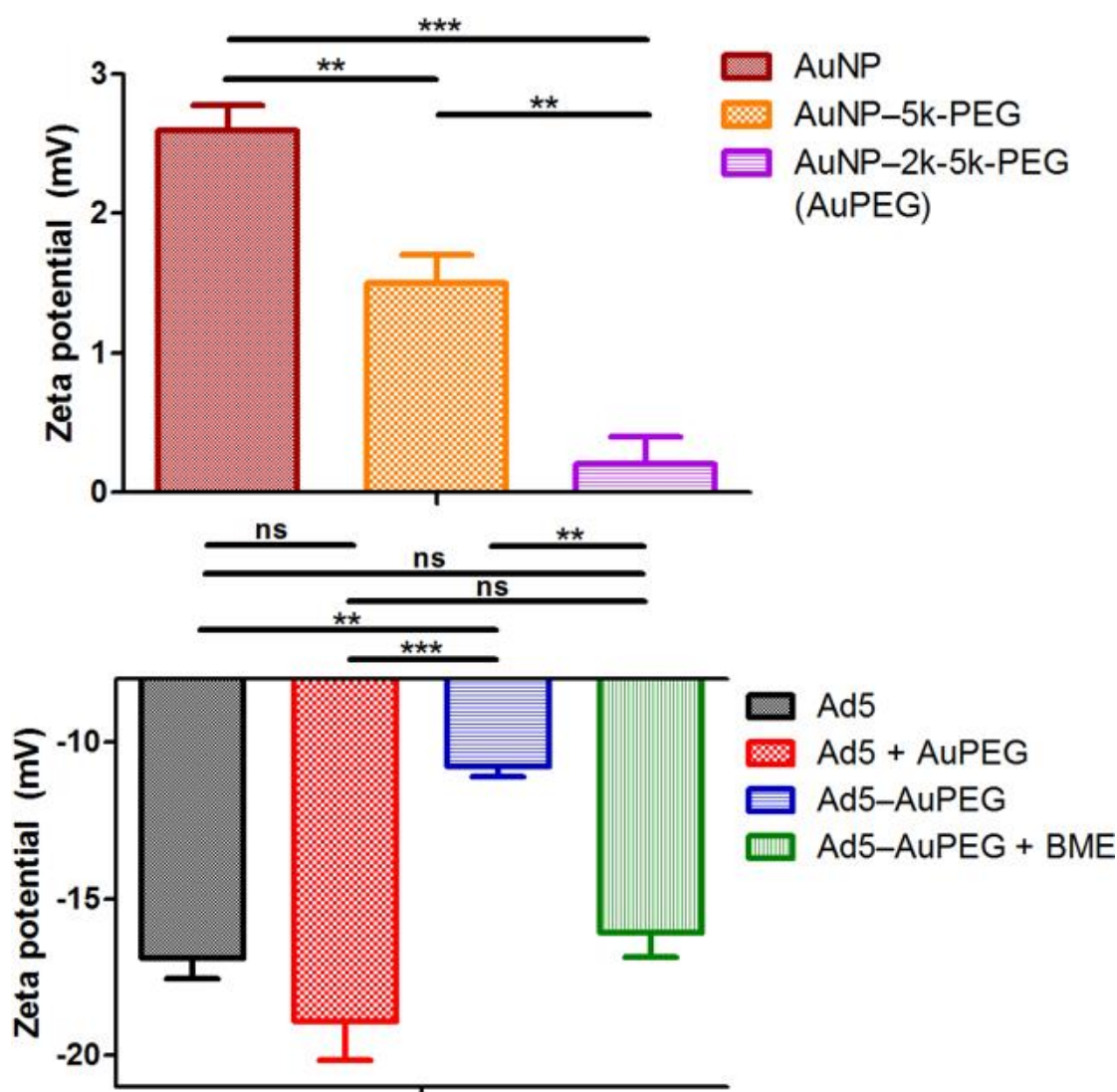


Figure 2.4: Influence of Au and Ad5 modification on particle surface charge. Zeta potential of each Au and Ad5 conjugation step was measured ($n = 4$, standard deviation shown). One-way ANOVA analysis was performed; *, **, and *** represents $p < 0.05$, 0.01 , and 0.001 , respectively. 'ns' indicates 'no significant' difference in the reported values.

	Zeta $\pm$ S.D. (mV)
Ad5	-16.9 $\pm$ 1.1
Ad5-PEG	-7.1 $\pm$ 0.22
Ad5-PHPMA	-0.14 $\pm$ 0.16
Ad5-AuPEG	-10.8 $\pm$ 0.54

Table 2.5: Zeta potential summary of Ad5, Ad5-PEG and Ad5-PHPMA and Ad5-AuPEG. Measured ζ -potentials (mean $\pm$ S.D.) of Ad5, Ad5-PEG and Ad5-PHPMA, and Ad5-AuPEG (n = 4) in PBS at 25°C.

The ζ -potentials of Ad5-PEG and Ad5-PHPMA (along with Ad5 and Ad5-AuPEG) are shown in **Table 2.5**. Although these three Ad5 modifications showed similar size (**Table 2.3**), their ζ -potentials were all significantly different from each other ($p < 0.001$). The most marked alteration of ζ -potential was achieved following addition of PHPMA (-0.14 mV). This is a multivalent reactive polymer conjugate and may be expected to produce a dense protective shield with polymer covering the negatively charged capsid more effectively than either PEG or AuPEG. These results demonstrated that the amine groups on Ad5 capsid were conjugated in much higher numbers to PHPMA than those on either Ad5-PEG (-7.1 mV) or Ad5-AuPEG (-10.8 mV) which rely on single point attachment of polymer constructs. While the exact quantification of PEG, PHPMA, and AuPEG molar ratio per Ad5 is discussed in section 2.5.5, the ζ -potential data imply that AuPEG achieved less Ad5 surface modification than PEG and PHPMA.

2.6.3 Results: SDS-PAGE and silver staining

In addition to the change in size and ζ -potential, alteration of the Ad5 capsid protein composition after stealthing with AuPEG was characterized by separating the Ad5 capsid proteins on a polyacrylamide gel. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a unique assay to separate a protein complex (e.g. Ad5 in this study) into its subunit proteins. Virologists use SDS-PAGE to determine if a specific protein of Ad5 has been chemically modified or not by examining if there is change in the molecular

weight of these proteins. If a protein gains molecular weight, it will ‘shift upward’ on a SDS-PAGE gel; similarly, if a protein loses its molecular weight, it will ‘shift downward’ on a SDS-PAGE gel. From the DLS and ζ -potential results, it is believed that some Ad5 capsid proteins were attached to AuPEG; as a result, by running a SDS-PAGE gel, this allowed the verification if such binding to capsid proteins (e.g. hexon, penton, and fibre) were successful or not.

Ad5 protein separation was performed on a 4% acrylamide stacking gel followed by 10% acrylamide separation gel, which matches the range (10 to 150 kDa) of polypeptides that constitute Ad5. First, the separation gel was made by mixing (in the following order) 4 mL of ddH₂O, 2.5 mL of 1.5M Tris-HCl (pH 8.8), 3.3 mL of acrylamide Bis (19:1 ratio; Sigma-Aldrich, UK), 100 μ L of 10% SDS (50 μ L), 50 μ L of 20% of ammonium persulfate (APS; Sigma-Aldrich, UK), and 100 μ L of tetramethylethylenediamine (TEMED; Sigma-Aldrich, UK) inside a 50 mL tube by gently rocking the tube back and forth; 6 mL of this freshly made separation solution were then poured immediately into a glass cast holder (0.75 cm x 10 cm x 10 cm). 200 μ L of 70% butanol was added on top of the separation gel (to push away bubbles and create an even horizontal border) and taken out after 45 min when the separation solution solidified into gel. Second, the stacking gel was made by mixing (in the following order) 6.1 mL of ddH₂O, 2.5 mL of 0.5M Tris-HCl (pH 6.8), 1.33 mL of acrylamide/Bis, 100 μ L of 10% SDS, 50 μ L of 20% of APS, and 100 μ L of TEMED inside a 50-mL tube by gently rocking the tube back and forth; 1 mL of this freshly made stacking solution were then poured immediately on top of the separation gel inside the same glass cast holder. Finally, a 10-well comb was submerged inside the stacking solution and allowed 30 min for setting.

2×10^9 copies of Ad5, Ad5–AuPEG, or non-linked Ad5 with AuPEG were incubated in 25 μ L of loading buffer containing 50 mM BME (**Figure 2.6:** lanes 1, 3, and 5) or equivalent volume of loading buffer with no BME (**Figure 2.6:** lanes 2, 4, and 6) for 5 min at 95°C before being loaded into the gel which was run for 90 min at 120 mV. The gel was then

stained with Bio-Rad Silver Stain Plus Kit (Bio-Rad Laboratories, US) in accordance with manufacturer's instructions. Molecular weights for all proteins were calculated using the protein ladder ColorBurst™ Electrophoresis Marker (8kDa to 220kDa; Sigma Aldrich, UK).

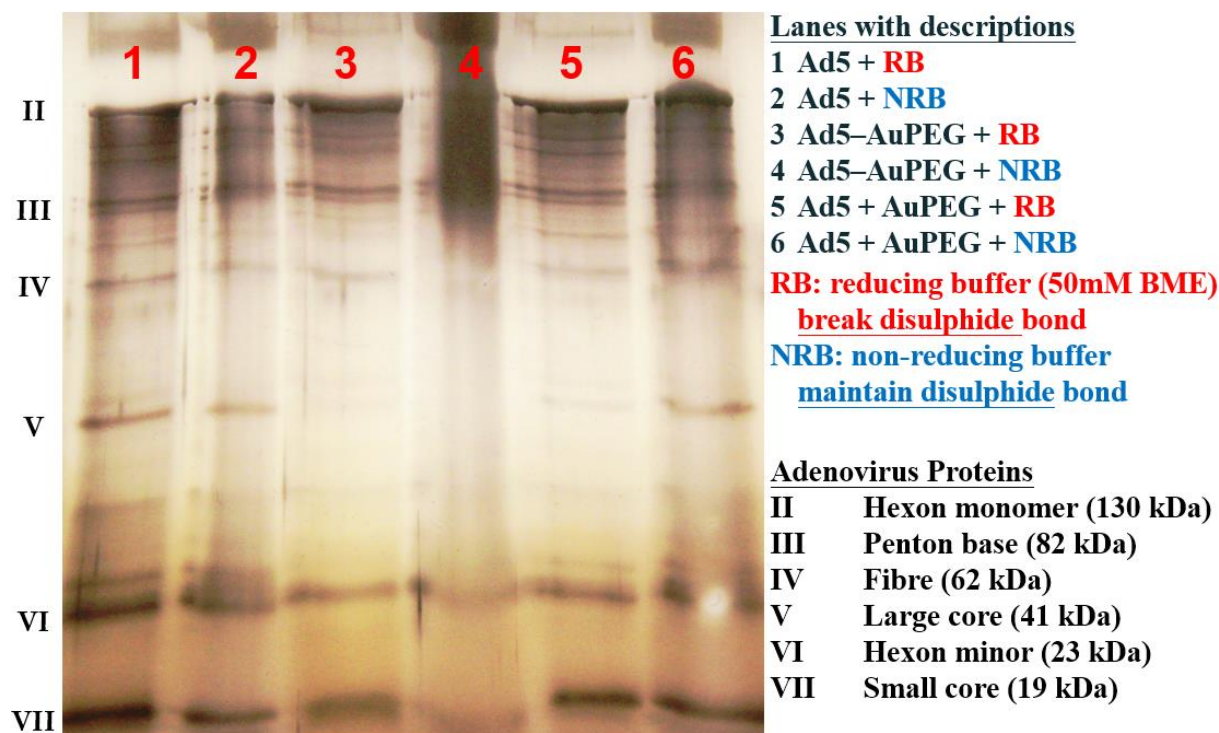


Figure 2.6: SDS-PAGE and silver staining characterisation of Ad5 protein modification with AuPEG. SDS-PAGE and silver staining was performed on Ad5, Ad5–AuPEG, and Ad5 + AuPEG in reducing buffer (RB; 50mM BME) or non-reducing buffer (NRB) as described in section 2.6.3.

The resulting SDS-PAGE silver stain (**Figure 2.6**) indicated that neither Ad5 nor non-linked Ad5 + AuPEG showed a difference in Ad5 capsid polypeptide band intensity or migration in the presence or absence of reducing buffer. In contrast, analysis of conjugated Ad5–AuPEG showed a dramatically different band migration pattern depending on the presence or absence of reducing buffer. Notably, in the absence of reducing agent (lane 4) there was little discernible migration of Ad5 capsid protein into the gel, indicating that most Ad5 capsid protein was bound to AuPEG and unable to properly penetrate the polyacrylamide. No bands were evident for Ad5 polypeptides II, III, and IV; notably the bands which did stain in lane 4 corresponded to the molecular weights of internal capsid proteins such as VI and VII [104]. However, upon exposure to reducing buffer, Ad5–AuPEG showed similar protein migration and intensity to that of Ad5 and non-linked Ad5 + AuPEG,

signifying the reduction-induced breakage of disulphide bonds between AuPEG and Ad5 to un-stealth Ad5 to its original form. While only one SDS-PAGE silver stain image is shown in this thesis, this experiment was repeated three more times and all images obtained looked similar to that shown in **Figure 2.6**. Ad5-PEG and Ad5-PHPMA were not analysed using the SDS-PAGE silver stain process because they did not have any reducible linkages between Ad5 and the coating. However, the DLS and ζ -potential data provided here were comparable to previous published results as discussed earlier, indicating that both Ad5-PEG and Ad5-PHPMA were successfully modified.

The reproducibility of the SDS-PAGE gel image for Ad5-AuPEG was 100%; this assay was performed three times, and every time a clear increase of capsid proteins' molecular weight was observed.

2.6.4 Results: transmission electron microscopy

To obtain visual evidence of AuPEG linked to Ad5 capsid, high magnification transmission electron microscopy (TEM) was used. 1×10^9 copies (in 5 μ L) of Ad5, Ad5-AuPEG, or non-linked Ad5 and AuPEG were deposited on formvar-coated 400 mesh copper grids (3.05mm diameter; TAAB Laboratories Equipment Ltd, UK) for 10 min; the grids were then stained with 5 μ L of 2% glutaraldehyde (Sigma-Aldrich, UK) for 5 min, washed with 5 μ L of ddH₂O twice, stained with 5 μ L of 0.5% uranyl acetate (Sigma-Aldrich, UK) for 1 min, and irradiated under the UV light (254nm) for 10 min before being imaged using a TEM machine (model A-7650; Hitachi, Japan). The TEM magnification was 70,000x at the accelerating voltage of 100 kV.

TEM images (**Figure 2.7**) provided visual evidence that many AuPEG were successfully bound to Ad5 (bottom right), whereas images of Ad5 + AuPEG (bottom left) showed that a few AuPEG were co-localised with Ad5. Using both TEM images and the molar extinction coefficient ($1.43 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$) of the Au particles, an average of

approximately 60 AuPEG per Ad5 capsid was calculated, and this number was significantly higher ($p < 0.001$) than the 5 to 10 AuPEG per Ad5 capsid in the Ad5 + AuPEG images. In addition, in the control Ad5 only images (top right), the 3-D contour of the triangular facets and hexons could be seen clearly; however, in the images taken for Ad5 + AuPEG and Ad5–AuPEG, such 3-D contour disappeared most likely due to the AuPEG’s interference with the electron beam. Furthermore, there was a substantial contrast between Ad5 capsid and Au particles since Au is so much denser than Ad5, so the electron beam was unable to focus on both the Au and Ad5’s surface at the magnification to give clear 3-D contour.

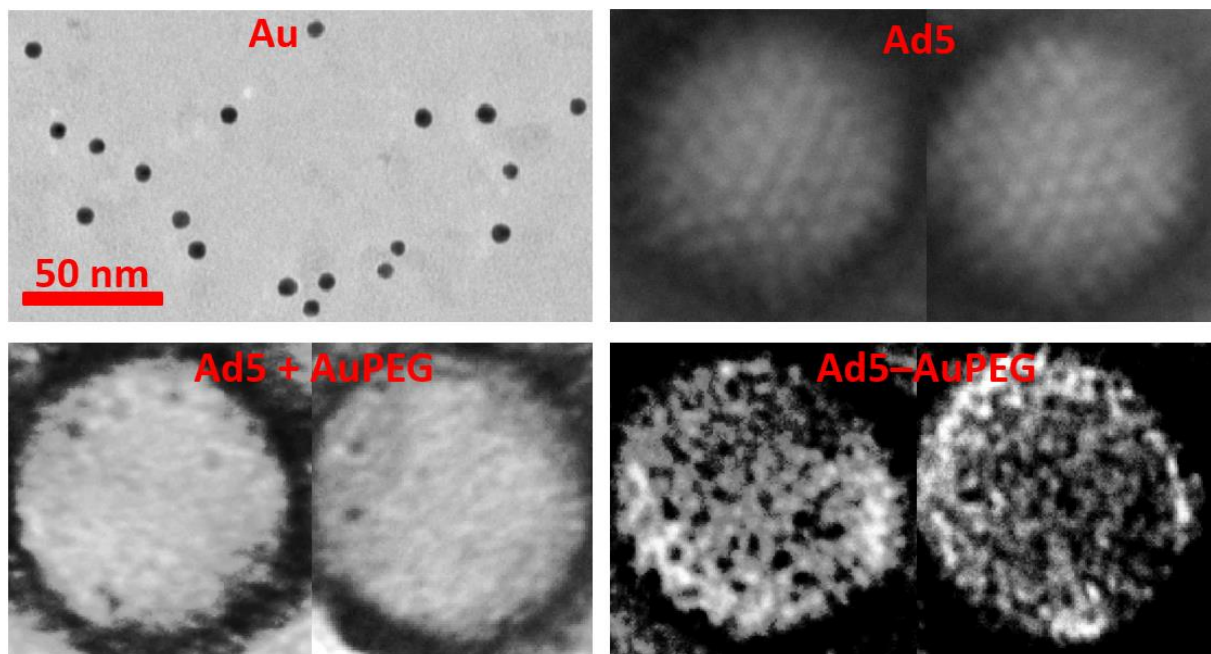


Figure 2.7: Visualization of Ad5–AuPEG binding by transmission electron microscopy. Transmission electron microscope was used to image unmodified Au, unmodified Ad5, Ad5 with AuPEG, and Ad5–AuPEG; the red scale bar represents 50 nm and applies to all four panels.

The Ad5 capsid core is 90 nm in diameter and has 20 equilateral triangular facets measuring 60 nm, if the 15 nm AuPEG spheres are perfectly aligned the absolute theoretical maximum number of AuPEG per facet is 6, giving 120 per capsid. However, the steric inhibition created by successful AuPEG binding makes it unlikely that such a number could be reached. Indeed, the TEM image allowed an average of 60 ± 8 AuPEG ($n = 20$) linked to each Ad5 capsid to be counted. It is important to note that because the 12 trimeric fibre proteins were lost from the Ad5 during TEM processing the AuPEG attached to these regions

cannot be visualised by this method. However, as SDS-PAGE (**Figure 2.6**) demonstrated that sufficient AuPEG was attached to the trimeric fibre proteins to prevent its migration it is therefore reasonable to assume that at least 3 AuPEG were attached to each of the 12 fibre trimers. Adding the capsid (60) and fibre values together (36) gives a total of approximately 96 AuPEG per adenovirus. Notably, TNBS analysis (section 2.6.5) demonstrated the loss of 111 amine groups from the Adenovirus surface upon reaction with AuPEG. These analyses, together, therefore indicate that the stealthing procedure enables the overwhelming majority of each of 96 AuPEG nanoparticles to be linked to adenovirus by just one bridging 5kDa PEG molecule.

2.6.5 Results: PEG quantification and density analysis of Au and Ad5 conjugates

Since Au and Ad5 have ~300 and ~1800 free amine groups, respectively, the quantification of the PEG density per Au and AuPEG density per Ad5 could be determined by measuring the free amine groups present on the Au and Ad5 pre- and post-modification. Although some studies have reported the utility of nuclear magnetic resonance and thermal gravimetric analysis (TGA) for the assessment of functionalization of Au [244], these studies could only show the change of physical and chemical properties instead of reporting a more meaningful molar ratio of conjugates to Au. Furthermore, TGA is more suitable for the study of hard metals and polymers but will not work on recognising the change of Ad5 surface modification since the heating process of TGA will permanently denature the viral protein without any mean to recover its structure and functionality.

Here an alternative approach of using a TNBS (2,4,6-trinitrobenzenesulfonic acid; Sigma-Aldrich, UK) assay was used to determine depletion of amine groups on both Au and Ad5 particles in response to addition of amine-reactive PEG, PHPMA or AuPEG [74]. In brief, two standard curves from known amine concentrations of unmodified Au or Ad5 in 50 μ L of sodium bicarbonate buffer (0.1 M, pH 9; Sigma-Aldrich, UK) were pipetted into a

transparent 96-well plate. The Au standard curve contained a maximum of 10^{12} particles, and the Ad5 standard curve started out with 10^{10} viral particles; one-half serial dilutions were carried out for both standard curves using the sodium bicarbonate buffer. 10^{12} copies of all the Au modifications and 10^{10} copies of all the Ad5 modifications synthesized in sections 2.3 and 2.4 were also placed in the 96-well plate. 50 μ L of 0.01% (w/v) TNBS solution was then mixed with all the sample wells. The reaction took place for 2 hr at 37°C, and 60 μ L of 10% SDS and 30 μ L of 1M HCl were then added to each well to stop the reaction. The plate was measured at 335-nm absorbance, and the standard curves were used to calculate the number of un-reacted amine groups per Au or Ad5 modification. These numbers of un-reacted amine groups were then subtracted from the total from un-modified Au and Ad5 to determine the surface modification percentage (**Table 2.8**) and the amount of PEG, PHPMA, and AuPEG per Au and Ad5 calculated. Since the density and molecular weight (MW) of all the chemicals and particles are already well-established in the literature, with the newly obtained data on amine depletion, the density and MW of all newly synthesised Au and Ad5 could be calculated (**Table 2.8**).

The TNBS assay confirmed the presence of an average of 6 x 5kPEG and 257 x 2kPEG per Au, lowering the density from 19.3 kg/L for Au to 15.5 kg/L for AuPEG and increasing the MW from 1.96 MDa for Au to 2.50 MDa for AuPEG. Although Au particles were saturated with 2kPEG, the very high density of AuPEG was still maintained, and this was of subsequent importance when AuPEG was conjugated to Ad5 to boost the overall Ad5–AuPEG density. The ratio of 2kPEG to 5kPEG was 42 to 1, indicating a close proximity to the proposed chemical structure in **Figure 2.1** with only a few 5kPEG per Au. The TNBS assay was used to calculate that an average of 111 free amine groups had been lost from the surface of Ad5 upon addition of about 96 AuPEG. Although only 95 PHPMA were attached per Ad5, it is important to note that each PHPMA polymer had 14 reactive TT groups with the potential to bind to Ad5. Therefore, TNBS analysis showed stealthing with Ad5–AuPEG was

achieved with modification of just 6% of total capsid amine groups compared to 74% with Ad5-PHPMA or 56% with Ad5-PEG. Notably, the loss of approximately 111 amine groups on the Ad5 capsid surface was in accordance with the attachment via a single point of the 60 Au visualised by TEM (section 2.6.4) plus the 36 that could not be visualised due to loss of fibre.

Modification	MW (g/mole)	Density (kg/L)	Conjugations	Surface Modified
Au	1.96 MDa	19.3	N/A	0%
Au–5kPEG	1.99 MDa	19.2	6 5kPEG per Au	2%
Au–5k-2kPEG	2.50 MDa	15.5	257 2kPEG per Au	88%
Ad5	150 MDa	1.37	N/A	0%
Ad5 + AuPEG	N/A	N/A	N/A	0%
Ad5–AuPEG	427 MDa	3.35	111 AuPEG per Ad5	6%
Ad5–AuPEG + BME	N/A	1.37	N/A	6%
Ad5–PHPMA	154 MDa	1.33	95 PHPMA per Ad5	74%
Ad5–PEG	170 MDa	1.35	1007 20kPEG per Ad5	56%

Table 2.8: Molecular weight (MW), density, conjugation ratios, and percentage of surface modified of Au and Ad5. Conjugation ratios were obtained from TNBS assay and enabled the subsequent calculation of percentage of surface modified, density, and MW of all Au and Ad5 modifications carried out in this study. N/A stands for data ‘not available.’

To characterise the density of Ad5–AuPEG, Ad5 and Ad5–PHPMA, ultracentrifugation through a caesium chloride (CsCl) density gradient was performed on these samples. In brief, a stock 500-mL 0.5M Tris buffer (pH 7.9) was first made; three CsCl density gradient buffers were made as follows: 1.32 kg/L (16.5 g of CsCl, 3.5 mL of the 0.5M Tris buffer, and 30 mL of ddH₂O), 1.45 kg/L (18.1 g of CsCl, 3.5 mL of the 0.5M Tris buffer, and 28.4 mL of ddH₂O), and 1.55 kg/L (19.4 g of CsCl, 3.5 mL of the 0.5M Tris buffer, and 27.1 mL of ddH₂O). A 40% glycerol stock solution was made by mixing 40 mL of glycerol, 2 mL of 0.5 Tris buffer, 1 mL of 0.1M EDTA, and 57 mL of ddH₂O. An ultracentrifuge tube (14 mL, 14 x 95 mm; Beckman, USA) was placed vertically and then 3 mL of 1.32, 1.45, and

1.55 kg/L CsCl buffers were injected sequentially from the bottom of the ultracentrifuge tube by a 25-gauge needle linked to a 5-mL syringe. 2 mL of the 40% glycerol stock solution was slowly placed on top of the 1.32 kg/L CsCl buffer. Two additional ultracentrifuge tubes were prepared with identical content described above. Lastly, 1 mL of PBS containing 10^{12} copies of Ad5, Ad5-PHPMA, or Ad5-AuPEG was placed on top of the 40% glycerol in the three ultracentrifuge tubes. The tubes were transferred into SW40 rotor tubes (Beckman, USA) which were then spun at 25,000g for 16 hr. This centrifuge speed and duration were based on trial and error testing.

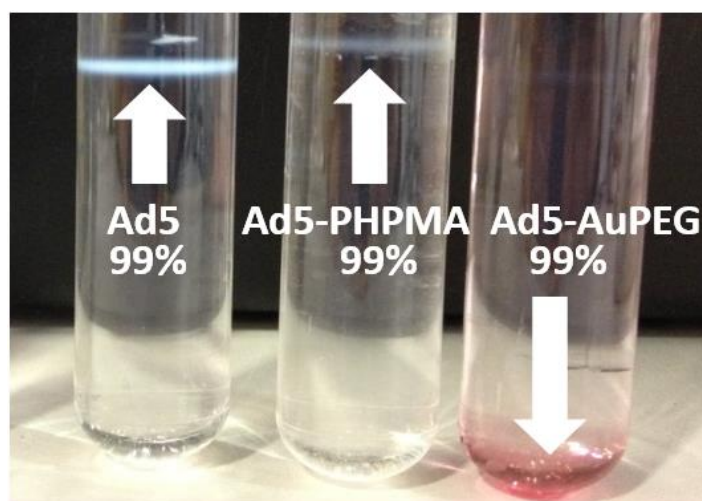


Figure 2.9: CsCl density gradient ultracentrifugation of Ad5, Ad5-PHPMA, or Ad5-AuPEG. CsCl gradient density buffers of 1.32, 1.45, and 1.55 kg/L were prepared inside the ultracentrifuge tubes overlaid with sample and spun at 25,000g for 16 hr. Ad5 and Ad5-PHPMA (both white bands) were retained at a density between 1.32 and 1.45 kg/L, while Ad5-AuPEG (red band) pelleted to the bottom below the 1.55 kg/L gradient buffer. qPCR demonstrated that the bands recovered from the three tubes contained > 99% of the sample originally loaded into the tube.

The results of ultracentrifugation are shown in **Figure 2.9**. Unmodified Ad5 has a defined density of 1.37 kg/L, compared to which the density of Ad5-PHPMA showed a slight decrease (shifting higher up the gradient). This is in accordance with previous analysis based on DLS size measurements and capsid modification % by TNBS assay (**Table 2.8**) which produced a value of 1.33 kg/L. As expected, the very dense Ad5-AuPEG sedimented to the bottom of the tube below the 1.55 kg/L, confirming its much increased density compared to Ad5. Although the density of Ad5-AuPEG was estimated to be 3.35 kg/L from the TNBS

assay, it was unfortunately impossible to make CsCl density gradient buffer at such density. The highest CsCl density gradients ever reported in the literature were of 1.7 and 1.8 kg/L for DNA and RNA purification, respectively [245]. An attempt to make CsCl density with concentration 1.9 kg/L or higher was unsuccessful since the CsCl powder was insoluble passing this concentration. Quantitative polymerase chain reaction (qPCR; see experimental methods in section 3.3.1) was performed to measure the Ad5 recovery in the top white bands from the Ad5 (> 99%) and Ad5–PHPMA (> 99%) tubes and the red band in the bottom of the Ad5–AuPEG tube (> 99%). DLS measurements further proved that the recovered Ad5, Ad5–PHPMA or Ad5–AuPEG had maintained its size and polydispersity, whose values were similar to those reported in **Figure 2.2**.

2.7 Summary and discussion

In this chapter, a new platform for stealthing nano-medicines is described whereby the exemplar nano-medicine Ad5 is conjugated to AuPEG to create a ‘dandelion’ structure. Ad5 capsid modification was confirmed and characterised using, DLS, ζ -potential, SDS-PAGE silver staining, TEM imaging, TNBS assay, and CsCl density gradient ultracentrifugation, to measure changes in size, surface charge, MW, and density. Having completed the characterisation of Au modification to AuPEG and Ad5 modification to Ad5–AuPEG the degree of stealthing provided by the AuPEG was then investigated. Furthermore, the efficacy of the reduction-responsive un-stealth mechanism was also confirmed.

Conjugating 20k-PEG (same MW as the PEG used in this thesis) to Ad5, Doronin *et al.* showed that the size of Ad5 increased from 110 nm for Ad5 to 138 nm for Ad5–PEG [103]; this 28-nm size increase was comparable to the 34-nm size measured in this study (**Table 2.3**). In addition, Morrison *et al.* reported the size increased from 118 nm for Ad5 to 135 nm for Ad5–PHPMA [246]; although this is slightly smaller diameter (135 nm) than the Ad5–PHPMA (145 nm) measured in this study (**Table 2.3**), Morrison *et al.* reacted Ad5 with

20 mg/mL PHPMA [246], whereas this study used 40 mg/mL. These findings suggested all Ad5 modifications used in this thesis produced a similar increase in size and were reproducible as compared with the literature standard. Unfortunately, neither Doronin nor Morrison reported the PDI for their Ad5-PEG and Ad5-PHPMA, respectively. However, judging from the width of size distribution of Ad5 and Ad5-PHPMA in Figure 1(b) of Morrison's paper [246], there was definitely an increase in PDI for Ad5-PHPMA compared to Ad5 alone. This finding was consistent with the increase of Ad5 (0.075) to Ad5-PEG (0.218), Ad5-PHPMA (0.203), and Ad5-AuPEG (0.194) shown in **Table 2.3**.

Although amine groups are positively charged and the depletion of the amine groups with PEG, PHPMA, or AuPEG conjugations suggested that the surface charge should become more negative, it was important to point out that amine was not the only functional groups on capsid surface of Ad5 (unlike the Au used in this study which was 100% coated with amine groups). For example, aspartic acid from the peptide RGD motif of adenovirus's penton base contributes to overall negative charge of Ad5 [247]. When PEG, PHPMA, and AuPEG covalently linked to the amine groups on Ad5, these large molecules also created steric hindrance to shield other negatively charged functional groups on capsid surface of Ad5, making the overall charge of Ad5 more positive and closer to neutral charge of 0 mV. The ζ -potentials reported in **Table 2.5** were comparable to those previously published in the literature. Alemany *et al.* demonstrated the ζ -potential increased from -10 mV for Ad5 to -6 mV for Ad5-PEG [88], while Seymour *et al.* showed the ζ -potential also increased from -25 mV for Ad5 to 0 mV for Ad5-PHPMA [248].

Subr *et al.* also previously used SDS-PAGE silver staining to demonstrate that Ad5-PHPMA had little discernible migration of Ad5 capsid proteins into the gel, indicating that most Ad5 capsid proteins were bound to PHPMA and unable to properly penetrate the polyacrylamide [106]. The SDS-PAGE silver staining image of Ad5-PHPMA by Subr and colleagues looked very similar as the image of Ad5-AuPEG on lane 4 of **Figure 2.6**; both

were in the absence of a reducing environment. In addition, both Subr's Ad5–PHPMA and the Ad5–AuPEG in this study showed no band for Ad5 capsid polypeptides II, III, and IV. More importantly, upon the exposure to Laemmli buffer (which contained BME), all the polypeptides of Ad5–PHPMA were once again as visible as those in the Ad5 control lanes of the gel [106]. These results suggested SDS-PAGE silver staining was a reliable assay to detect the covalent surface modification of Ad5 for both Ad5–PHPMA and Ad5–AuPEG.

By combining the DLS and zeta potential data (**Tables 2.3 and 2.5**), it was confirmed that the tumour-mimicking reducing environment created by BME was able to cleave AuPEG off Ad5. Regarding the transformation from Ad5–AuPEG back to Ad5, the decrease in size from 149 nm (Ad5–AuPEG) back to 120 nm (Ad5–AuPEG + BME) and the decrease in zeta potential from -10.8 mV (Ad5–AuPEG) to -16.1 mV (Ad5–AuPEG + BME) confirmed such reducing environment could re-activate Ad5–AuPEG back to Ad5, despite the probable presence of a few thiol adducts. However, whether this successfully triggered release had any biological utility was yet to be discussed. Thus, these biological characterisations are reported in the following chapter. Lastly, the discussion of how the size of Ad5 and Ad5–AuPEG fit into the wider context of nano-medicine *in vivo* delivery in animal studies and further clinical trials is addressed in Chapters 5 and 6.

CHAPTER 3

IN VITRO STUDIES OF THE BIOLOGICAL INTERACTIONS OF AD5–AUPEG

3. *In vitro* studies of the biological interaction of Ad5–AuPEG

The biological consequences of the changes detected by the physicochemical analyses described in Chapter 2 were assayed using an enzyme-linked immune sorbent assay (ELISA), infection of cancer cell lines, and binding to human red blood cells.

The aims of this chapter were to:

- 1) Compare how Ad5–PEG, Ad5–PHPMA, and Ad5–AuPEG reduce anti-Ad55 antibody binding compared to un-modified Ad5.
- 2) Investigate if the reduced Ad5–AuPEG could still infect cells and the percentage of cells that could be infected by Ad5–AuPEG.
- 3) Determine if the hexon protein of Ad5–AuPEG was shielded properly by measuring the influence of FX mediated infection.
- 4) Examine if Ad5–AuPEG could prevent binding to human erythrocytes.
- 5) Discuss the abundant literature for Ad5–PEG and Ad5–PHPMA and how these findings might be suitable for Ad5–AuPEG going into *in vivo* work.

3.1 Enzyme-linked immune sorbent assay (ELISA) assay

ELISAs can reliably and sensitively determine the binding of antibodies to protein(s) of interest (e.g. Ad5 capsid proteins). The purpose of ELISA in this study was to compare whether the binding of anti-Ad5 antibodies to Ad5 decreased upon modification with PEG, PHPMA, or AuPEG. Polyclonal rabbit anti-adenovirus antibody raised against whole intact capsid (ab6982-100; Abcam, UK), was diluted 1/500 in PBS; 50 μ L of the diluted antibody was added to each well of a Nunc-Immuno Maxisorb flat-bottom 96-well plate (Fisher Scientific, UK), and the plate was sealed with a transparent microplate standard adhesive foil (Greiner Bio-one, US). After incubation over night at 4°C, the plate was washed 10 times with PBS using a Nunc-Immuno Wash 12 (VWR, UK). After 50 μ L of blocking buffer (95%

PBS + 5% heat inactivated goat serum, HINGS, from Sera Lab, UK) was added to each well, the plate was sealed and incubated at 37°C for 1 hr, then washed twice with PBS.

Ad5, Ad5 + AuPEG, Ad5–AuPEG, Ad5–PEG, and Ad5–PHPMA samples were diluted to between 1.6×10^8 and 1×10^7 particles per well in diluent (95% PBS + 5% HINGS) and 50 μ L added to each well; each Ad5 modification had four repeats ($n = 4$) for each concentration. The samples were sealed and incubated for 2 hr at 25°C before the plate was washed 10 times in PBS. Polyclonal goat anti-adenovirus-biotin conjugate (0151-9104; AbD Serotec, UK) was diluted 1/100 in diluent, and then 50 μ L was added to each well and sealed and incubated at 25°C for 1 hr. After washing 10 times in PBS, 50 μ L of avidin- horseradish peroxidase (18-4100-94; eBioscience, UK) diluted 1/1000 in diluent was added to each well and sealed and incubated at 25°C for 30 min. After washing 10 times in PBS, 50 μ L of tetramethylbenzidine substrate (80091; Alpha Diagnostic International Inc., US) was added to each well and left to develop at 25°C for 10 min. 50 μ L of stop solution (80100; Alpha Diagnostic International Inc., US) was added to each well. All ELISA plates were read at 450 nm, 1 sec on Wallac Victor² 1420 multi-label counter (PerkinElmer, USA). Absorbance values in this study are represented after subtraction of the value obtained for the negative background control. One Way ANOVA Newman-Keuls multiple comparison tests were performed for all samples at the same concentration; the results were represented by *, **, and *** which stand for $p < 0.05$, 0.01, and 0.001, respectively.

ELISA results from **Figure 3.1** show that antibody binding to Ad5 and Ad5 + AuPEG were significantly higher ($p < 0.001$) than the level of binding to Ad5–AuPEG. The non-statistically significant positive control (Ad5 + AuPEG) further strengthened the argument that AuPEG does not interfere with Ad5 when not covalently linked to the virus capsid. In addition, **Figure 3.2** compares the ELISA binding results across Ad5, Ad5–PEG, Ad5–PHPMA, and Ad5–AuPEG. While there was no significant difference ($p > 0.05$) between Ad5 and Ad5–PEG in terms of antibody binding (only 10% reduction), both Ad5–PHPMA and

Ad5–AuPEG demonstrated much lower ($p < 0.001$) antibody binding than Ad5 or Ad5–PEG. Indeed, the absorbance reading achieved with 1.6×10^8 Ad5–AuPEG was equivalent to the signal achieved using 1×10^7 copies of Ad5 or Ad5 + AuPEG, indicating the binding of antibodies had been reduced by 90%. When similar analysis was made, 1.6×10^8 Ad5–PHPMA produced a signal equivalent to that achieved using 4×10^7 copies of Ad5 or Ad5 + AuPEG, showing that its antibody binding had been reduced by 75%. While both Ad5–PHPMA and Ad5–AuPEG provided an impressive level of stealthing of the Ad5 capsid, the 90% reduction in binding for Ad5–AuPEG is still significantly lower ($p < 0.01$) than the 75% reduction in binding for Ad5–PHPMA. A strong correlation between the ELISA binding results and *in vivo* circulation half-life data is described in Chapter 5 of the thesis.

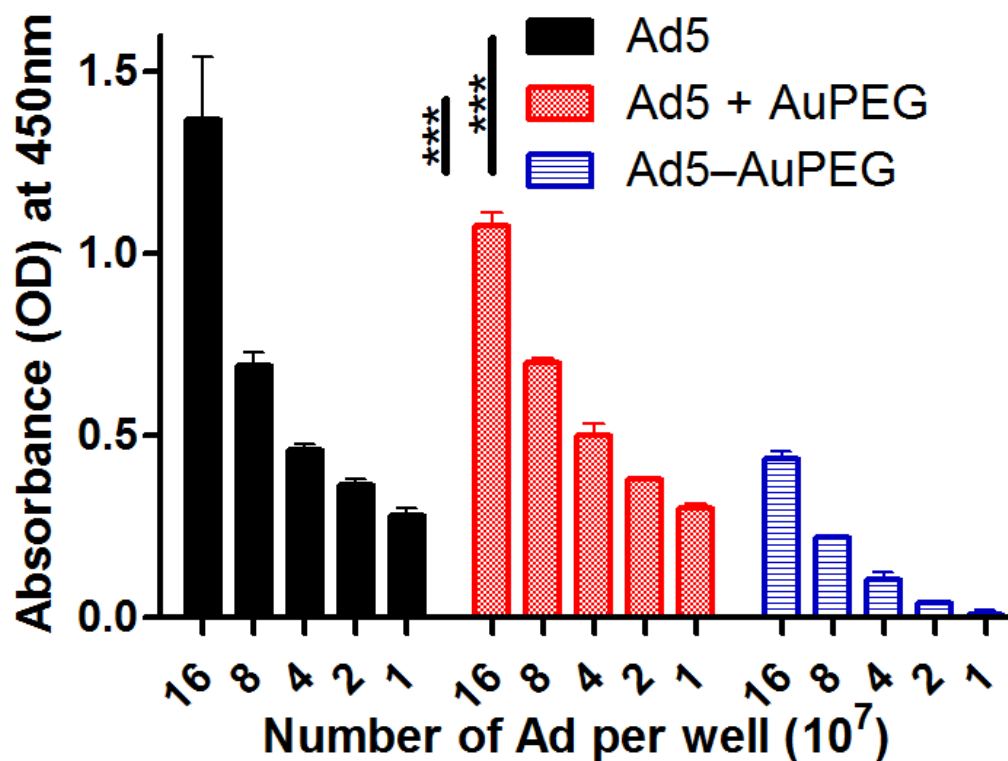


Figure 3.1: Consequences of coating for protection against anti-Ad5 antibody binding. Two-fold serial dilutions of sample were made and an ELISA performed. Ad5 = un-modified Ad5, Ad5 + AuPEG = control non-linked Ad5 and AuPEG, and Ad5–AuPEG = chemically conjugated Ad5 and AuPEG. N = 4, S.D. shown. Analysis by one-way ANOVA, *** = all groups $p < 0.001$ for all Ad5 concentrations.

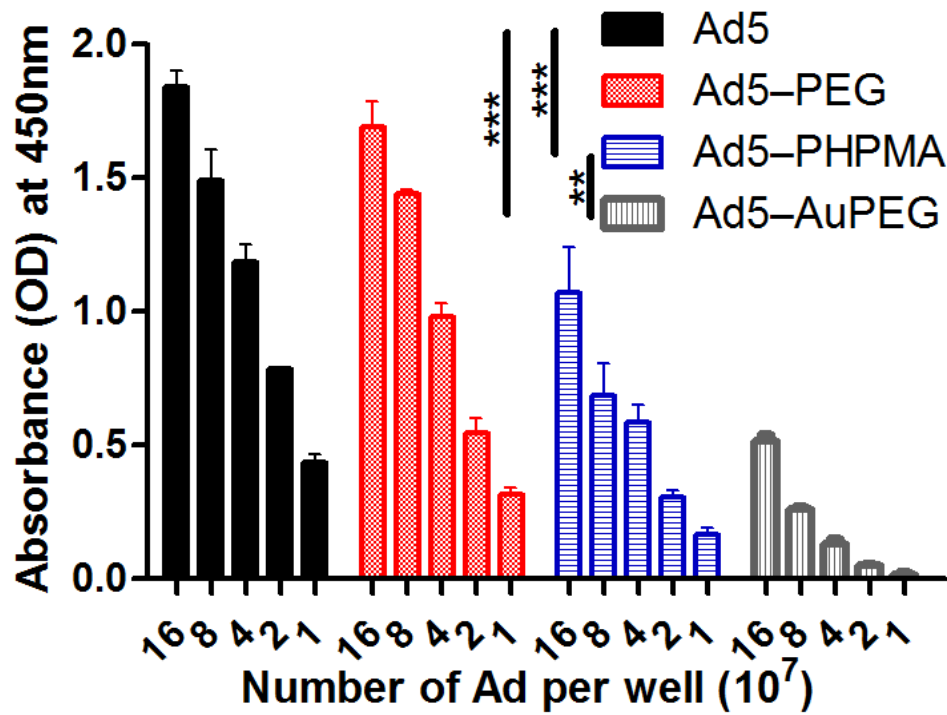


Figure 3.2: Consequences of coating for protection of Ad5-PEG, Ad5-PHPMA, and Ad5-AuPEG binding against anti-Ad5 antibody binding. Two-fold serial dilutions of sample were made and an ELISA performed. N = 4, S.D. shown. Analysis by one-way ANOVA, ** and *** represent all groups $p < 0.01$ and 0.001 , respectively, for all Ad5 concentrations.

3.2 Cell infection assays

After confirming the decrease in antibody binding of Ad5-AuPEG, *in vitro* assessment of its cancer cell line infection efficiency was carried out. Human ovarian cell lines IGROV-1 and SKOV-3 cells (European Collection of Cell Cultures, Salisbury, UK) were cultured in high glucose Dulbecco's modified Eagle's Media (DMEM; PAA Laboratories, UK) containing 2 mM glutamine (PAA Laboratories, UK), supplemented with 1% penicillin-streptomycin solution (pen/strep; Sigma-Aldrich, UK) and 10% fetal bovine serum (FBS; PAA Laboratories, UK). The cells were cultured on T-175 flask (PAA Laboratories, UK) at 37°C with 5% CO₂, and the cell culture media was changed every 72 hr. The rationale for the use of IGROV-1 and SKOV-3 was that although they are both of ovarian origin, but IGROV-1 cells express normal/high level of coxsackievirus and adenovirus receptor (CAR) while SKOV-3 cells express low level of CAR. In fact, by running a flow cytometry to detect the interaction of RmcB (a CAR monoclonal) with these two cell lines, the CAR expression level was 12-

fold lower in SKOV-3 than in IGROV-1. This differential CAR expression therefore permitted the influence of stealthing on fibre-CAR interactions as well as hexon-factor X (FX) interactions to be assayed as a means of characterising the efficiency with which these different capsid regions were stealthed. Effective coating of hexon protein has proven particularly challenging due to its net negative charge [246].

After a critical cell confluency was reached, IGROV-1 or SKOV-3 cells were plated on clear tissue culture 96-well plates (PAA Laboratories, UK) at 20,000 cells and 100- μ L cell media per well. All cell infection procedures (described below) were carried out 24 hr after cell plating to allow time for cell adhesion. After removing cell culture media, four wells of IGROV-1 cells were infected with Ad5 or Ad5–AuPEG (1,000 v.p. per cell), which had been exposed to PBS containing BME ranging from 0 mM to 10 mM for 20 min prior to the cell infection. The infection media consisted of 50 μ L of the PBS with Ad5 or Ad5–AuPEG and 50 μ L of DMEM + 10% FBS. The rationale of using 10mM BME had been discussed in section 2.3.

The influence of FX and other serum proteins on hexon mediated infection and therefore the degree of hexon stealthing can be determined by including FBS (which includes trace amount of FX, according to the manufacturer) or excluding it from the infection media. Hence, while all IGROV-1 cells were transduced with DMEM + 10% FBS, the low CAR SKOV-3 cells were used the infections were performed with +FBS media or –FBS media. With a slight modification of the experimental setup, four wells of SKOV-3 cells were transduced with Ad5 or Ad5–AuPEG (1,000 v.p. per cell), which had been exposed to PBS containing no BME or 10 mM BME for 20 min prior to the cell infection. Half of the infection media consisted of 50 μ L of the PBS with Ad5 or Ad5–AuPEG and 50 μ L of DMEM + 10% FBS, while the other half consisted of 50 μ L of the PBS with Ad5 or Ad5–AuPEG and 50 μ L of DMEM.

Both IGROV-1 and SKOV-3 cells were then incubated at 37°C for 90 min with 5% CO₂ to allow viral infection. The infection media was removed from the wells, and 100 µL of cell culture media (DMEM with 10% FBS and 1% pen/strep) added. Cells were incubated at 37°C overnight, washed twice with PBS, and removed for flow cytometry analysis. All Ad5 used in this project (with the exception of the survival study in Chapter 5) was E1 deleted and encoded the GFP reporter gene. Cells were analysed using a flow cytometer (FACS Calibur; Becton Dickinson, UK) equipped with a 488-nm air-cooled argon-ion laser. Fluorescence was detected using 520 nm (FL1) and 575 nm (FL2) band-pass filters for GFP. Background fluorescence and auto-fluorescence were determined using mock-treated IGROV-1 and SKOV-3 cells. Cellular debris showing reduced side and forward scatter was excluded from further analysis and also defined as unhealthy or unviable. 10,000 events were acquired per sample (n = 4). CellQuest Pro analysis software (BD Biosciences) was used to analyse the percentage of GFP-expressing cells and GFP mean fluorescence intensity (MFI) per positive cell. All statistical analyses in this section was completed using a one-way analysis of variances (ANOVA) followed by a Newman-Keuls test for pairwise comparison of sub-groups. The IGROV-1 and SKOV-3 results are representative of three and two repeat experiments respectively.

3.2.1 Analysis of IGROV-1 cell infection

Ad5 infection results in IGROV-1 cells are shown in **Figure 3.3** as the percentage of total infected cells and in **Figure 3.4** as the mean fluorescence intensity (MFI) per positive cell. The result from IGROV-1 cells (which express high levels of CAR) showed dramatically reduced infection activity for Ad5–AuPEG to virtually 0%, indicative that good stealthing of the fibre domain which binds to CAR had been achieved, in accordance with the SDSP-PAGE analysis in section 2.6.3. Furthermore, the data showed that there was a ‘switch on’ at 0.5-mM BME whereupon 20% of the IGROV-1 cells were infected, yet when the maximum 10 mM BME was reached (which was designed to mimic the tumour-like reducing

environment [232, 241, 242]), 42% of the IGROV-1 cells were infected. For the cells treated with un-modified Ad5 in both the absence and presence of BME, 90% infection was achieved. The full recovery of infection activity was not possible despite BME treatment providing complete removal of AuPEG and thereby returning the Ad5–AuPEG to the size of un-modified Ad5 (as shown by DLS data in **Figure 2.2**). This may be due to the presence of thiol adducts which remain on the capsid post-cleavage of the AuPEG and may inhibit the ability of the virus to bind to cellular receptors [62]. Notably, the MFI of cells successfully infected with 10 mM BME treated Ad5–AuPEG (4400 AU, absorbance unit) was not significantly different ($p > 0.05$) from the MFI values of un-modified Ad5 (4200 AU) in the presence and absence of BME, indicating those 42% of IGROV-1 cells infected by Ad5–AuPEG + 10-mM BME were equally amenable to infection by Ad5–AuPEG as those 90% of IGROV-1 cells infected by the control Ad5. However, the MFI values for Ad5–AuPEG with 0.5-mM to 5-mM BME were only, at best, half the value of 10-mM BME (ranging from 200 to 2000 AU), showing that at lower BME concentrations, the AuPEG cleavage from Ad5 was sub-optimal Ad5. In addition, cell viability data showed that $> 98\%$ of all infected IGROV-1 were healthy, demonstrating that exposure to these levels of Ad5 or Ad5–AuPEG and 10 mM BME did not cause cell toxicity. Incubation of Ad5 with 10 mM BME for over 30 min was shown to decrease un-modified control Ad5 infectivity (measured by GFP expression level from Ad5 transduction) by more than 20% (data not shown); as a result, the 20 min time point was chosen as a standard throughout the remaining experiments. One hypothesis was that after 30 min, BME interact with Ad5 capsid proteins to create possible steric hindrance, reducing their ability to bind to host cell to trigger the same level of infection as shown in the absence of BME.

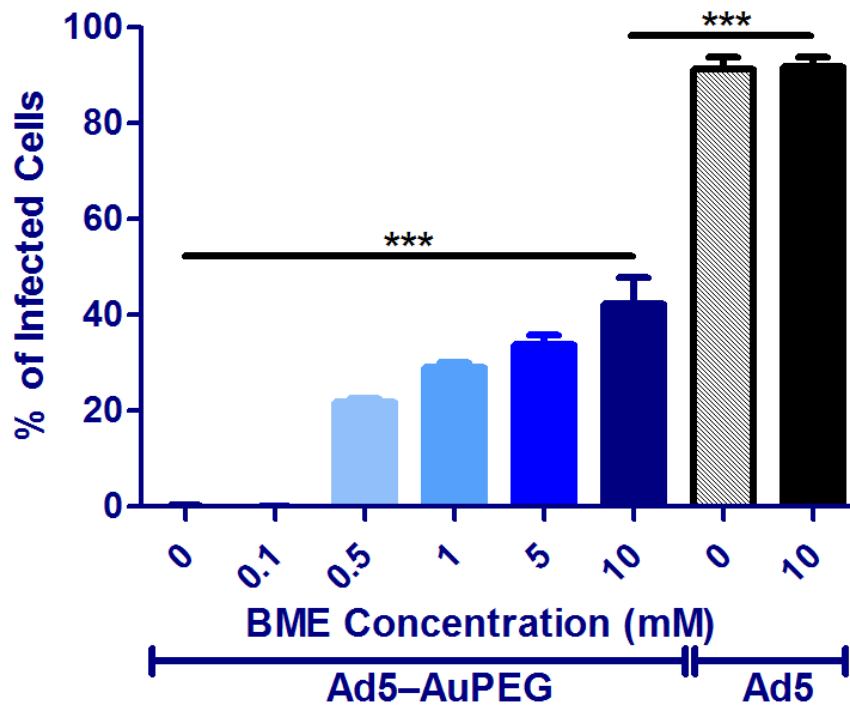


Figure 3.3: The influence of BME concentration on re-activation of Ad5–AuPEG infection of IGROV-1 cells. The utility of the reduction sensitive cleavage and un-stealth mechanism was demonstrated by infecting IGROV cells with Ad5 or Ad5–AuPEG following their pre-incubation with a range of concentrations of the reducing agent BME. Percentage of cells positive of GFP transgene expression is shown. N = 4, S.D. shown. Groups compared using one-way ANOVA; *** represents p-value < 0.001.

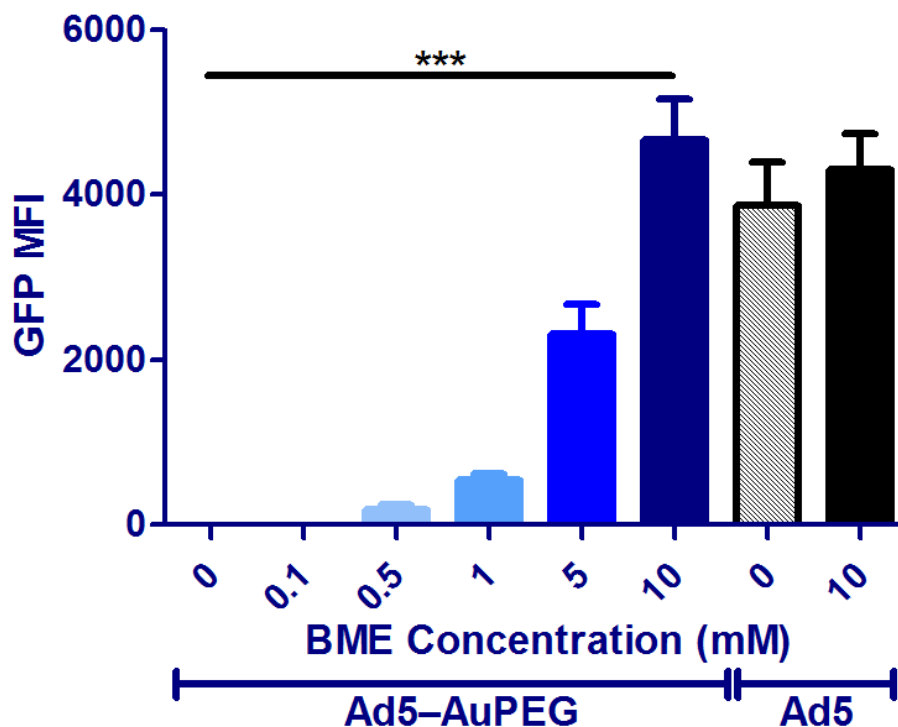


Figure 3.4: The influence of BME concentration on re-activation of Ad5–AuPEG as assessed by the mean fluorescence intensity (MFI) of GFP transgene expression in IGROV-1 cells. This figure corresponds to the percentage of infected IGROV-1 cells in Figure 3.3, showing the MFI for each sample. N = 4, S.D. shown.

3.2.2 Analysis of SKOV-3 cell infection

Figures 3.5 and 3.6 show the experimental data from the SKOV-3 infection, which are low in CAR and are therefore infected via FX and other serum protein mediated interactions with the hexon protein of Ad5 [64, 249]. In these cells, the level of infection with Ad5 in the presence of FBS (45%) was 4-fold greater than in the absence of FBS (11%), in line with previous reports [64].

In contrast, Ad5–AuPEG showed little response to FBS addition, with levels of 0.06% in the presence of FBS and 0.05% in its absence (**Figure 3.5**), indicating hexon regions were effectively stealthed. However, following BME addition infection with Ad5–AuPEG was restored in the presence of FBS to provide 8.7% positive cells compared to 0.44% in the absence of FBS. This demonstrates that un-coating allowed FBS mediated infection to be restored proving that hexon regions were effectively coated by AuPEG and uncoated by BME.

In addition, every single group with FBS infection cell media had approximately 4-fold more ($p < 0.05$) GFP positive SKOV-3 cells following Ad5 or Ad5–AuPEG infection compared to the same group without FBS in the infection cell media. In contrast to IGROV-1 cells which produced GFP MFI values which increased with increasing BME concentration, all SKOV-3 cells infected by Ad5 or Ad5–AuPEG had consistent GFP MFI at around 290 AU ($p > 0.05$) as shown in **Figure 3.6**. Cell viability data showed that $> 98\%$ of all SKOV-3 cells were healthy, demonstrating that 10 mM BME did not cause cell toxicity.

Notably, despite both cell lines being exposed to the same amount of Ad5 for the same amount of time, IGROV-1 and SKOV-3 cells showed different maximal GFP MFI levels. For IGROV-1 cell infection peaked at around an MFI of 4,000 AU, whereas the MFI of SKOV-3 peaked at around 300 AU. The highest percentage of infected SKOV-3 was 44% for control un-modified Ad5 in DMEM with +FBS infection media, a much lower level than the 90%

achieved for IGROV-1 using the same infection conditions ($p < 0.05$). These findings are the result of the much lower CAR expression in SKOV-3 cells [64, 249].

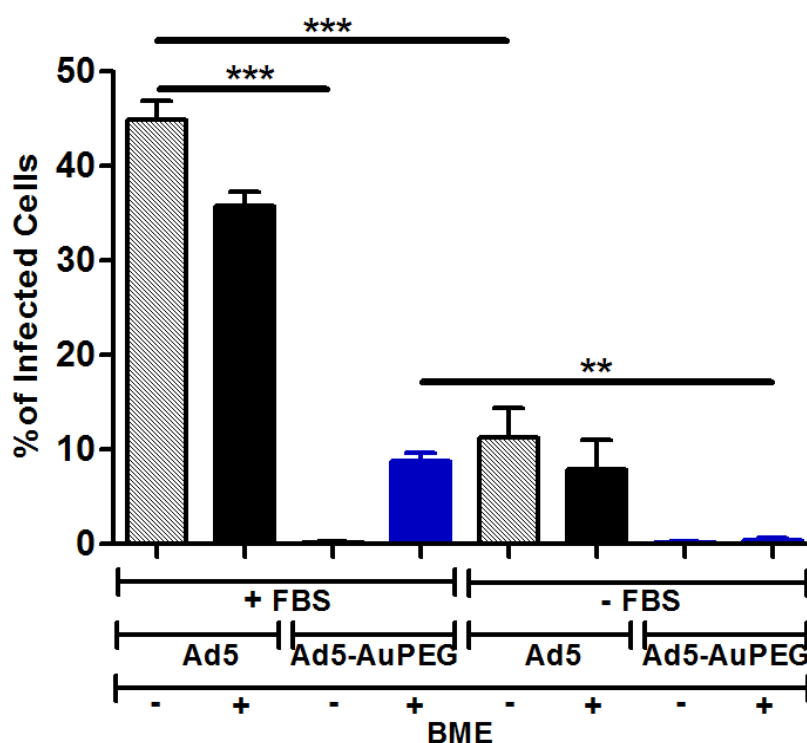


Figure 3.5: Influence of FBS on SKOV-3 cell infection by Ad5 and Ad5-AuPEGs. All BME used in this setup were 10 mM. Since SKOV-3 cells are CAR-deficient and therefore dependent on FBS for Ad5 infection; the failure of FBS addition to enhance Ad5-AuPEG activity demonstrates that Ad5 FBS binding regions in hexon protein are well protected as a result of AuPEG modification. N = 4, S.D. shown.

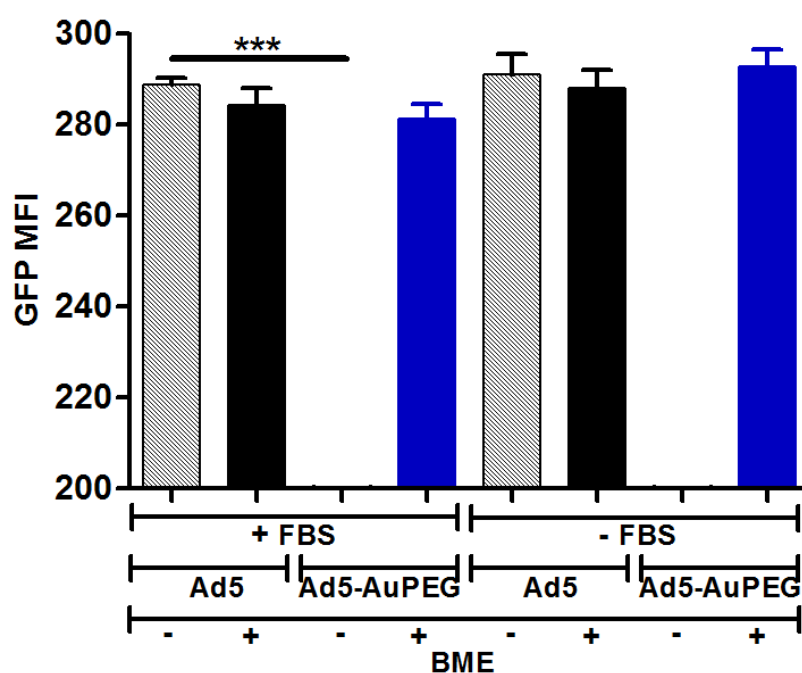


Figure 3.6: The mean fluorescence intensity (MFI) of GFP expression in infected SKOV-3 cells. GFP positive SKOV-3 cells in Figure 3.5 were assessed for their MFI. N = 4, S.D. shown.

3.3 Blood cell binding to Ad5 vs. Ad5–AuPEG

A study by Carlisle *et al.* has demonstrated that human, but not murine, erythrocytes may have a function in preventing systemic Ad5 infection by acting as circulating ‘virus traps’ [250]. The authors raised a crucial question regarding whether using mice for testing intravenous Ad5 therapeutics is a good model to predict the actual pharmacokinetic behaviour of systemic Ad5 in human. The paper showed that more than 90% of the Ad5 bound to human erythrocytes after only 30 min of interaction [250].

In order to address the same question and to test the ‘shielding’ of Ad5 by AuPEG, a similar experiment was carried out by incubating 10^9 copies of Ad5 or Ad5–AuPEG with 1 mL of human blood ($n = 4$) at 37°C for 30 min and measuring the viral particles remaining free in the blood plasma. Human blood sample was taken from a healthy volunteer (female, age 26). After the incubation period, the samples were centrifuged at 1,000 $\times g$ for 5 min. This relatively low centrifugal force was used to try and prevent the much denser Ad5–AuPEG co-sedimenting with the cell pellet. The supernatant (blood plasma) was purified for Ad5 DNA and the amount of recovered Ad5 calculated using quantitative polymerase chain reaction (qPCR) as described in section 3.3.1.

3.3.1 Purification of Ad5 DNA for quantitative PCR analysis

This section is a general protocol for purification of adenovirus DNA followed by qPCR analysis. Many parts of the DPhil thesis will refer back to this section when describing the quantification of Ad5 from various mouse tissues, blood plasma, and *in vitro* tumour-mimicking materials (e.g. phantom). All samples tested for Ad5 genome content were purified by using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, UK). In brief, 200 μ L of blood plasma or homogenised tissue samples (dissolved in PBS at 125mg/mL) were mixed with 200 μ L of Lysis Solution C (which contained proteinase K, a powerful enzyme which digests proteins and also inactivates nucleases [251]) and incubated

at 55°C for 1 hr for protein digestion and then at 70°C for 20 min to inactivate proteinase K. The sample was mixed with 200 µL of 70% ethanol and immediately transferred into a GenElute™ Miniprep Binding column, which had first been washed with 500 µL of GenElute™ Column Preparation Solution. The column was then spun at 12,000 x *g*, washed through twice with 500 µL of GenElute™ Wash Solution, and then the Ad5 DNA eluted by addition of 200 µL of GenElute™ Elution Solution. The purified Ad5 genome could now be stored at -20°C for later assay or immediately be assessed by qPCR.

Amplification of an 84-bp fragment of the adenovirus fibre gene was carried out using the primers 5' TGG CTG TTA AAG GCA GTT TGG 3' and 5' GCA CTC CAT TTT CGT CAA ATC TT 3' with detection of amplified sequences by a TaqMan probe (5' TCC AAT ATC TGG AAC AGT TCA AGT GCT CAT CT 3'), which was labelled at the 5' end with the FAM fluorophore and at the 3' end with the TAMRA quencher. FAM is a commonly used fluorophore with a 520-nm emission wavelength. Primers and probe were purchased from Sigma Genosys (now part of Sigma-Aldrich, UK). Reactions were carried out in a total volume of 25 µL in qPCRBIO Probe Mix (PCR Biosystems Ltd, UK) containing primers and probes at concentrations of 400 and 200 nM, respectively. All non-DNA-containing components were UV irradiated for 15 min prior to reactions being set up in a sterile environment. Thermocycling parameters were optimised as 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 95°C (30 sec) and 60°C (2 min). Analysis of data was carried out using the StepOnePlus™ software (Invitrogen, UK), and test samples were compared to standards of known Ad5 DNA content based on PicoGreen analysis (as described in section 2.5). Standard curves were prepared for individual tissue types by spiking serial dilutions of known Ad5 concentration into control tissues and extracting DNA for qPCR analysis as described above.

3.3.2 Blood cell binding of Ad5-AuPEG

The efficient stealthing of Ad5 by AuPEG was confirmed by qPCR which measured the total Ad5 and Ad5–AuPEG remaining in human plasma (**Figure 3.7**).

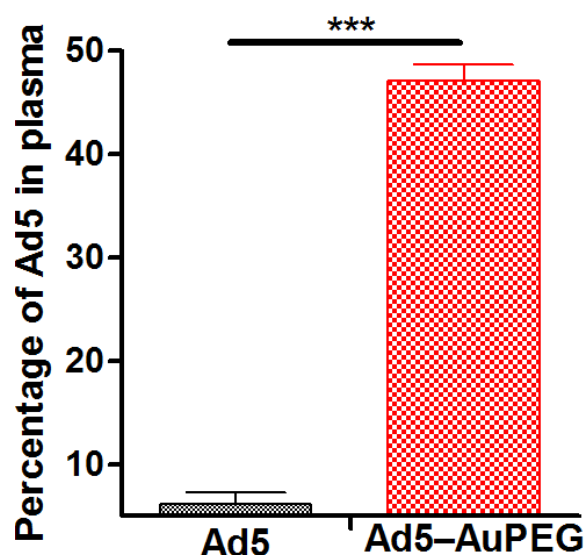


Figure 3.7: Influence of AuPEG stealthing on Ad5 binding to human blood cells. Ad5 or Ad5–AuPEG was incubated with fresh human blood for 30 min. The binding was then assessed by separating the plasma and cell fractions by centrifugation and performing qPCR specific for the Ad5 genome on the blood plasma. Data are represented as the percentage of the total input dose recovered. N = 4, S.D. shown; *** represents $p < 0.001$.

The results demonstrate that Ad5–AuPEG (47%) had >10-fold lower ($p < 0.001$) binding to human blood cells than Ad5 (4.2%). The percentage of Ad5 recovered from human blood plasma was comparable to the 3.6% reported in the previous study [250]. The actual amount of Ad5–AuPEG in blood plasma could potentially be higher since the centrifugal force used to separate blood and plasma might sediment some of the dense Ad5–AuPEG with the cell pellet. A small-scale experiment performed by spinning Ad5–AuPEG inside PBS at the same gravitational force ($1000 \times g$) and time (5 min) showed a small fraction of Ad5–AuPEG (< 5%) did form a pellet. However, since human blood is much denser and richer in proteins than PBS, a direct ‘back calculation’ of the actual loss of Ad5–AuPEG due to centrifugal spinning could not be calculated. A conservative estimation of at least 10% of Ad5–AuPEG was lost due to centrifuge would put the total Ad5–AuPEG remained in human blood plasma to be around 60%. These results, together, demonstrate that the Ad5–AuPEG

shielding is much more protective against human erythrocytes binding than un-modified Ad5, making Ad5–AuPEG more clinically applicable.

3.4 Summary and discussion

This chapter investigates the various biological effects of AuPEG stealthing of Ad5. Comparison is made to control Ad5 and in some cases Ad5–PEG and Ad5–PHPMA. In brief, failure to bind to anti-Ad5 antibodies, failure of FBS to enhance SKOV-3 cell infection, and the prevention of binding to human erythrocytes are good indicators of tropism ablation [58].

The ELISA experiment showed that Ad5–AuPEG had dramatically decreased binding to antibodies (90%) than Ad5 and was much more effectively shielded than both Ad5–PEG (10%) and Ad5–PHPMA (75%). Fisher *et al.* reported a 80% drop in antibody binding to Ad5–PHPMA compared to un-modified Ad5 [117], and this was in accordance with the 75% reduction binding of Ad5–PHPMA reported in **Figure 3.2**. Interestingly, although several studies have synthesised Ad5–PEG for various *in vitro* experiments, there are no published data showing the effects of its ELISA antibody binding. This thesis is the first to report such findings and also, for the first time, directly compare the antibody binding of Ad5–PEG and Ad5–PHPMA to show Ad5–PHPMA has superior protection against antibody neutralisation than Ad5–PEG. Lastly, these findings have important repercussions for the applicability of this technology in humans as pre-existing anti-Ad5 antibodies are present in about 85% of the population [91] and even if not present would be expected to be generated in response to initial dosing of patients in clinical trials.

Infection of high-CAR IGROV-1 cell cells established that stealthing with AuPEG could essentially completely ablate infection, which indicates good coverage of Ad5 fibre proteins in accordance with SDS-PAGE analysis. Notably, only 42% of cells could be infected upon reactivation of Ad5–AuPEG by exposure to tumour-like reduction potential. O'Riordan and colleagues were the first group to synthesise Ad5–PEG and show that both

protection against neutralisation and maintenance of 45% infection could be achieved [110], which is perhaps a rather contradictory finding. On the other hand, Fisher *et al.* first reported the coating of Ad5 with PHPMA and demonstrated an impressive knockdown of native tropism; the authors showed that using VEGF as a re-targeting ligand could enhance Ad5–PHPMA infection to specific cells. Compared to un-modified Ad5, Ad5–PHPMA had a 10-fold decrease in transgene expression while Ad5–PHPMA–VEGF gave a 2-fold increase in transgene expression in HUVE cells [117].

As reviewed earlier in Chapter 1, to achieve highly efficient targeted delivery with Ad5, both receptor binding (e.g. CAR/integrins) and bridging interactions (e.g. FX) are crucial. In mice, this FX ‘bridge’ connects the binding with membrane surface-expressed heparin sulphate proteoglycans (HSPG) on hepatocytes to trigger the liver transduction. Both mechanisms were crucial to ablate liver infection in an *in vivo* study [67]. More importantly, recent studies have reported that in the absence of CAR, HSPG still permits the binding of Ad5 [92, 93]. In this study, infection of low-CAR SKOV-3 cells in the presence and absence of FBS showed that the hexon protein of Ad5 was effectively stealthed since the percentages of infected SKOV-3 by Ad5–AuPEG without FBS and Ad5–AuPEG with FBS were 0.05% and 0.06%, respectively (**Figure 3.5**). Comparing this result to previous studies, Ad5 coated with 5k-PEG still led to high levels of FBS-mediated hepatic infection, but Ad5 coated with 20k-PEG and 35k-PEG were able to avoid such interference [103, 252]. Wisse *et al.* suggested that the sizes of un-modified Ad5 and Ad5–PEG_{5k} were below the murine endothelial fenestration area cut-off at 140nm, yet since Ad5–PEG_{20k} and Ad5–PEG_{35k} were measured above this size, their liver tropism was greatly reduced [253]. This was a reason why 20k-PEG was used in this DPhil study as a comparator instead of smaller PEG for coating Ad5. A more detailed discussion of the difference between murine and human endothelial fenestrae and the implications for clinical practice are presented in Chapter 5.

Carlisle and colleagues demonstrated that human, but not murine, erythrocytes may have a function in preventing systemic Ad5 infection by acting as circulating ‘virus traps’. Interestingly, the same study also reported that human erythrocytes express high level of CAR and that CAR was not expressed in murine erythrocytes [250]. This finding has a very important implication for the translation of murine model to clinical trial for virotherapy. In this thesis studies using whole fresh blood, compared to Ad5, Ad5–AuPEG showed 10-fold reduced binding to the cell fraction (**Figure 3.7**). This reinforces that good coating of fibre can interact with CAR on erythrocytes, and that the remainder of the capsid which can be a target for antibodies leading to sequestration by erythrocytes and leukocytes. While there is no literature reporting on the interaction between human erythrocytes with Ad5–PEG yet, Subr *et al.* demonstrated a significantly lower interaction between human erythrocytes and Ad5–PHPMA [106]. In addition, human (but not murine) erythrocytes also express complement receptor, which is essential in binding Ad5 in the presence of antibodies and complement [250].

In addition to matching or exceeding the stealthing achieved with alternative established PEG or PHPMA strategies [88, 97, 103], the Ad5–AuPEG described here was designed to achieve tumour responsive de-stealthing. To date there has been just one previous report where such a de-stealthing mechanism of Ad5 has been included: Subr and colleagues linked Ad5 to PHPMA also via a disulphide bond [106]. This similarly reducible design significantly lowered the interaction of Ad5 with erythrocytes from 95% down to 5% (compared to 95% down to 40% for Ad5–AuPEG in this study) and decreased the influence of FX mediated infection by a factor of 2 in SKOV-3 cells (compared to a 5-fold decrease for Ad5–AuPEG). However, the authors did not show what percentage of normal high-CAR cells could still be infected by the reduced form of Ad5–PHPMA [106], so no direct comparison could be made with the 42% recovered IGROV-1 infection percentage reported

in this study. Whether this cleavable Ad5–PHPMA has any improved therapeutic effect *in vivo* compared to conventional non-reducible Ad5–PHPMA is yet to be reported.

In addition, the disulphide-based conjugations have been a popular choice for nano-medicine delivery; many of these applications include both protein-based and oligonucleotide-based delivery [231]. Wen *et al.* synthesised doxorubicin-loaded nano-micelles which were conjugated to PEG via a disulphide bond and utilised this design to test its drug release *in vitro*. Under the tumour-like reduction environment (10 mM GSH), the nano-micelles were able to release the drugs and decrease the cell viability of MCF-7 breast cancer cells from 95% down to 20%; when GSH was absent, the nano-micelles were only able to decrease the cell viability from 95% down to 45% [254]. Luo and colleagues conjugated collagen to mesoporous silica nanoparticles (which entrapped FITC as model drug) via disulphide linkage; under DTT reducing environment, 65% of the FITC molecules were released inside HepG2 cells compared to only 12% of FITC release in the absence of DTT [255]. Compared with these two studies, the 42% infected cells by Ad5 upon re-activation reported in this thesis had a similar release level. These are just two of hundreds of papers published using disulphide bond as a triggered mechanism to release anti-cancer nano-medicines from their carriers.

It could be argued the use of glutathione (GSH) instead of BME is more appropriate for *in vitro* infection reactivation studies. In this thesis, BME was chosen because there is literature (see section 2.3) directly correlating tumour extracellular reducing potential (-260 mV) with a BME concentration of 10 mM. This concentration of this agent was chosen to specifically demonstrate that the trigger mechanism is appropriate to the reducing potential present within the extracellular milieu in tumours. GSH is a protein found within the cytoplasm whereas the strategy for Ad5–AuPEG reactivation relies on cleavage of the AuPEG in the reducing extracellular environment thereby allowing virus reactivation and cell infection via its highly evolved pathway. GSH is therefore not relevant to such a scenario.

Furthermore, the reduction potential of cytoplasmic GSH has been reported to be between -180 and -160 mV [256], which was substantially less reducing than that value of -260 mV reported for the extracellular environment of tumour interstitium [232, 242].

Interestingly, Ad5-PEG and Ad5-PHPMA are able to infect tumours in murine models when infection is ablated *in vitro* and no mechanism for un-coating is provided. This may be because incomplete coating of fibre could allow CAR or HSPG interaction. It is more likely that incomplete coating of hexon regions permits FBS mediated infection *in vivo*. This is not observed *in vitro* because knockdown experiments are routinely performed in high CAR cells and FBS is not specifically tested for. The degree of polymer and/or nanoparticle modification of v.p. can be assessed by analysing the changes in size, surface charge, and antibody neutralisation ability. These data should be reported not only to allow the validation of the coating mechanisms but also assist the prediction of plasma circulation and liver infection in pre-clinical testing in animal models [58].

The chemical characterisation and the biological interactions of Ad5-AuPEG in Chapters 2 and 3, respectively, permit further investigation of how this powerful stealthed agent can be combined with therapeutic ultrasound to investigate the second hypothesis of this project: increasing the density of a therapeutic particles (Ad5 in this study) using gold nanoparticles enhances response to ultrasound-induced microstreaming to enhance extravasation deeper into the tumour.

CHAPTER 4

***IN VITRO* STUDY ON THE ACOUSTIC EFFECTS OF GOLD-COATED ADENOVIRUS**

4. *In vitro* study on the acoustic effects of gold-coated adenovirus

The first experiments aimed at enhancing, understanding and controlling the acoustic response of Ad5 were carried out by Bazan-Peregrino *et al.* [159]. A phantom vessel running through agar gel containing breast cancer cells was developed as an *in vitro* 3D tumour model for these studies. The oncolytic adenovirus Ad5EHE2F-Luc used in the study can selectively replicate in cells that experience hypoxia and/or express estrogen receptor and was previously shown to be the best agent among a variety of oncolytic adenovirus for breast cancer treatment [45]. Ad5EHE2F-Luc was introduced through the phantom vessel, and a focused 0.5 MHz HIFU transducer was used to instigate acoustic cavitation in the presence and absence of the ultrasound contrast agent (UCA) SonoVue®. While delivering the same amount of acoustic energy, the ultrasound exposure parameters were optimised to maximise either the ultra-harmonic content (360 kPa, 90% duty cycle and PRF of 10 Hz for stable cavitation) or broadband spectral content (1.25 MPa, 6.5% duty cycle and PRF 10 Hz for inertial cavitation) of the acoustic emissions [159]. Both stable and inertial cavitations are described in section 1.3. In the absence of ultrasound, very few cancer cells were successfully infected by the oncolytic virus. However, the instigation of cavitation correlated with increased adenovirus extravasation resulting in increased infection efficacy. Interestingly, small increases in the total amount of adenovirus transferred into the agar led to marked increases in the transgene expression achieved. Stable cavitation caused a two-fold increase in the number of viral particles extravasated, which translated into a 10-fold increase in expression at 24 hours. Most impressively, inertial cavitation caused a four-fold increase in viral concentration that translated into a 200-fold increase in luciferase expression. These interesting findings have since been validated in murine models [45].

Building on these early observations, a key hypothesis of the present work is that increasing the density of Ad5 with the conjugation of gold nanoparticles to produce Ad5–AuPEG would result in more extensive extravasation relative to un-modified Ad5 or polymer-

coated Ad5–PHPMA under inertial cavitation conditions. Assuming that the Ad5, Ad5–PHPMA, and Ad5–AuPEG are able to reach their terminal velocities under cavitation events, the densest Ad5–AuPEG should possess the greatest momentum since all three viral particles are similar in size.

In order to test this hypothesis, a precisely controlled tumour-mimicking environment needed to be created in terms of: vessel diameter, tumour mass, flow rate, and pressure built up from the environment. Animal models were not deemed suitable for these preliminary studies because of the major differences in vascularity and vascular permeability even amongst animals of the same gender, species, implanted tumour type, and weight. In addition, since Ad5, Ad5–PHPMA, and Ad5–AuPEG will mostly likely have different blood circulation half-lives due to different shielding effects against blood interactions (see Chapter 5), it would be difficult to ensure that the same amount of viral particles (v.p.) were present in tumours across experiments.

The aims of this chapter were to:

- 1) Develop an *in vitro* tumour-mimicking flow-channel model to test the proposed hypothesis.
- 2) Compare and analyse the extent of Ad5 extravasation under stable versus inertial cavitation.
- 3) Determine the best Ad5-modified therapeutic based on the depth and amount of extravasation for *in vivo* study.
- 4) Visualise the pattern of Ad5 extravasation under no ultrasound, stable cavitation, and inertial cavitation.
- 5) Conclude if increasing the density of Ad5 could provide more extensive extravasation under identical inertial cavitation conditions.

4.1 *In vitro* experimental setup and data acquisition

In order to enable mechanistic testing of the hypothesis, the optimal experimental model would allow the same amount of Ad5, Ad5–PHPMA, or Ad5–AuPEG passing through the same tumour-mimicking structure at the same flow rate to be exposed to identical ultrasound conditions. A tumour-mimicking flow-vessel model was thus developed to meet these criteria and test the hypothesis *in vitro*. After many failures on other models and surveying many possible phantom models, an OptiCell® holder was chosen as the mould for the following reasons: firstly, it enabled the convenient creation of two small flow channels of diameter 1 mm; secondly, the acoustically transparent windows allows seamless propagation of the ultrasound field in the axial direction; thirdly, cells can be embedded in a safe and sterile environment; fourthly, fluorescence microscope imaging can be performed directly on the OptiCell® holder to provide good visualization of the Ad5 extravasation pattern without the need to sample or risk losing spatial registration of the ultrasound-exposed regions.

This section is divided into three sub-sections describing methods for the manufacturing of an OptiCell® flow-channel model, the ultrasound setup, and the actual ultrasound parameters and Ad5 therapeutics injected into the flow channels.

4.1.1 Design of OptiCell tumour-mimicking flow phantom model

To achieve capillary-like vessels in the phantom, a mould was first built by drilling two 1.0-mm-in-diameter stainless steel rods through an OptiCell® holder (Thermo Fisher Scientific, USA), which was a sterile 74 mm x 65 mm x 3 mm cast with acoustically transparent windows on both sides (**Figure 4.1**). The two stainless steel rods were 25 mm apart. Each rod was located 20 mm from the top and bottom edges of the OptiCell®.



Figure 4.1: Opticell mould model used to create the tumour tissue-mimicking phantom. Two 1-mm stainless steel bars are used to create flow-channels in the phantom after agar embedded with cancer cells were injected into the mould.

Ovarian cancer IGROV-1 cells were cultured as previously described in Section 3.2. UltraPure™ Agarose-1000 (Invitrogen, UK) was microwaved in PBS, cooled to 37°C and then mixed with culture media and cells to give a final concentration of 0.5% agarose and 5% FBS in a final volume of 50% PBS and 50% DMEM. IGROV-1 cells were incorporated in the agarose liquid at a final concentration of 5×10^5 cells/mL. For sterilisation, the OptiCell® holder (which still contained the two stainless steel rods) was exposed to UV light (254-nm wavelength) for 10 min. 20 mL of this suspension was injected into each OptiCell® holder inside a tissue culture hood to maintain sterile condition and allowed to set for 30 min at 4°C to form a porous gel of approximately 600-1200 nm pore diameter [257] which is similar size to the endothelial gap of subcutaneous tumours [258]. The two stainless rods were later removed to create 1-mm flow channel inside the OptiCell® holder. Two sides of the OptiCell® holder were made of acoustically transparent windows to allow the entrance and exit of the ultrasound field in the axial direction.

The four side-openings of the two flow channels on each OptiCell® were each connected to a 5-cm 25G vacutainer (Becton Dickinson, UK) by 1mL of hot melt adhesive (Hobby Craft, UK). The other end of each vacutainer was connected to 50-cm polyvinyl chloride tubing (inner diameter 0.5 mm; outer diameter 1mm; Thermo Fisher Scientific, USA) by hot melt adhesive (**Figure 4.2**). The construct was allowed to rest at room

temperature for 10 min to allow gelling of the agar/cell matrix and setting of adhesives before submersion and ultrasound exposure.

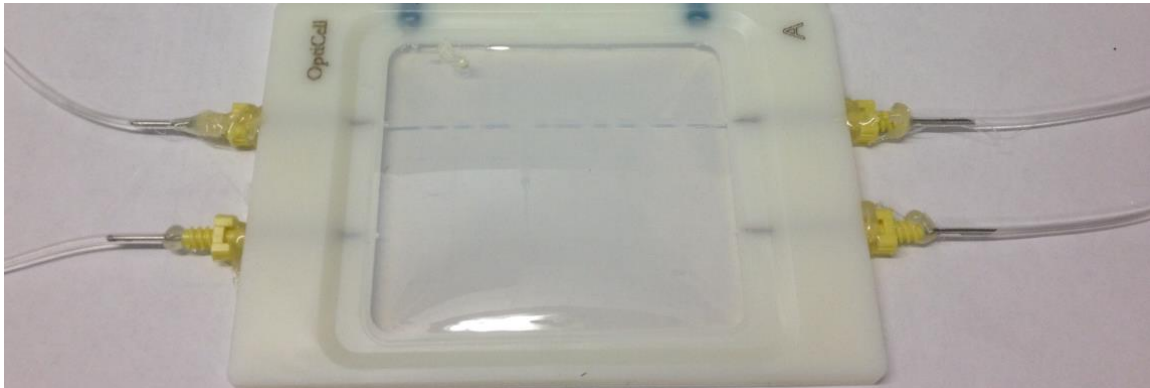


Figure 4.2: Tumour tissue-mimicking flow-channel phantom. Each flow-channel in the phantom was connected to two a vacutainer which was then linked to a 50-cm polyvinyl chloride tubing with inner diameter 0.5 mm and outer diameter 1 mm.

4.1.2 Ultrasound apparatus for *in vitro* experimentation

The experimental procedures described in this section were carried out in collaboration with two highly skilled researchers, Dr. Eleonora Mylonopoulou and Susan Graham. In particular, their development and optimisation of the equipment setup and ultrasound parameters employed in this study, as well as their expertise with therapeutic ultrasound, were instrumental to the success of these experiments.

In this *in vitro* study, HIFU was generated by a single-element spherically focused ultrasonic transducer with a centre frequency of 508 kHz and 150 kHz bandwidth (H-107D; Sonic Concepts, USA). The custom made software using Labview 10.0 (National Instruments, UK) was used to control the HIFU. The computer controlled a waveform generator (33250A; Agilent Technologies, USA) whose signal was amplified by 55 dB using a 300W RF power amplifier (A-300; Electronic Navigation Industries, USA). The matching network of the HIFU transducer was subsequently connected to the power amplifier. The 508-kHz transducer was operated only at its centre frequency with a geometric focus of 63 mm. The beam profile of the HIFU focus has a full-width half-maximum of 30 mm axially and 3 mm transversally.

The beam profile around the HIFU focus was characterised using a 0.075-mm element diameter needle hydrophone (Precision Acoustics, UK). An absolute pressure calibration was also performed using the same hydrophone, and all pressures reported throughout this DPhil study are peak rarefactional focal pressures (PRFP).

Acoustic emissions were recorded passively with a 15 MHz spherically focused transducer (V319; Panametrics, UK) used as a passive cavitation detector (PCD). The PCD was placed in the 2-cm central opening of the HIFU transducer, and these were confocally and coaxially aligned as previously described [259, 260]. The raw signal from the PCD was amplified 25-fold (SR445A; Stanford Research Systems, USA) and high-pass filtered (model 3950; Krohn-Hite, USA) at 2 MHz to reject reflections of the driving frequency, first and second harmonic of the 508 kHz transducer. Data was recorded at sampling rate of 20 MHz with an 8-bit digitizer (Picoscope 3205; Pico technology, UK).

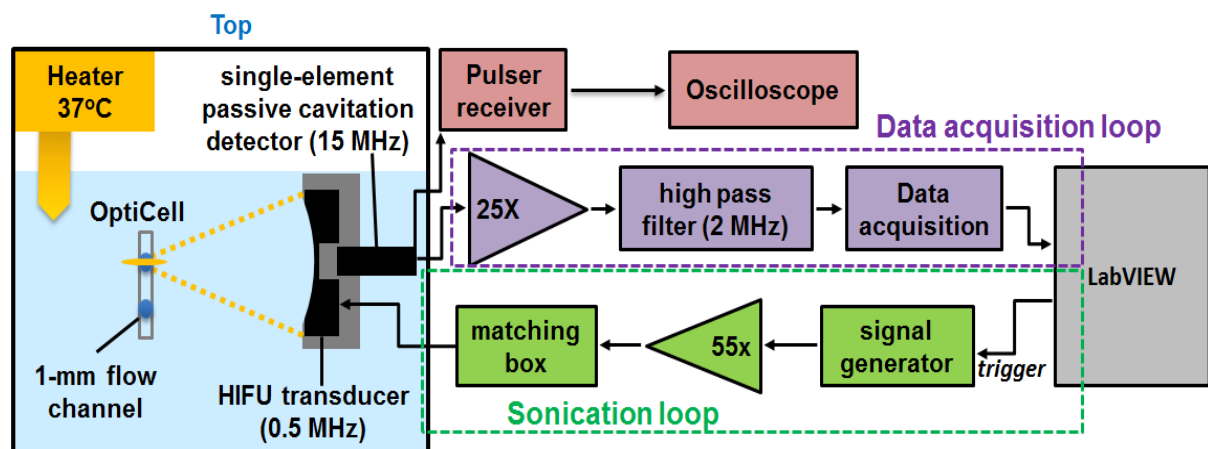


Figure 4.3: Block diagram of the experimental apparatus. The driving signal for the HIFU transducer is generated by a computer-controlled signal generator and a 55 dB high-power RF amplifier, followed by a transducer-specific matching network to optimise power transfer. Meanwhile, acoustic emissions from the focus are measured using a passive cavitation detector (PCD) aligned confocally and coaxially with the HIFU transducer. Before being digitized and recorded by the data acquisition unit in the computer, the signal from PCD amplified 25-fold and then conditioned using a 2MHz high-pass filter. Also shown are an ultrasonic pulser/receiver and an oscilloscope, which are used to align the OptiCell® phantom with the transducers but are disconnected during exposures.

The focus of the HIFU was always aligned to the centre of either the top or bottom channel of the OptiCell phantom using pulse-echo imaging of some sample SonoVue®

microbubbles flowing through the flow channels. Alignment was carried out using an ultrasonic pulser/receiver (DPR300; JSR Ultrasonics, USA) operating in pulse-echo mode, as well as a digital oscilloscope (1 GHz bandwidth, 8-bit, Wavesurfer 104Mxi; LeCroy, UK). Because the same OptiCell phantom used for the alignment was subsequently tested for the experiment on the same channel, there might be potential pre-conditioning of the channel by the microbubbles; however, later cavitation results showed this was not a concern.

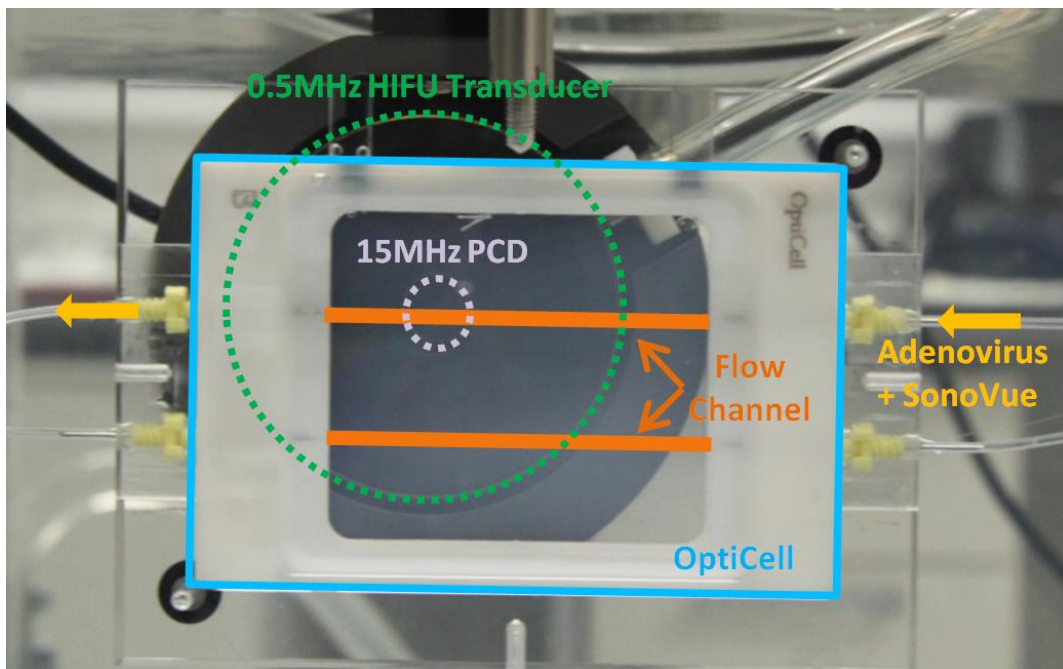


Figure 4.4: Front view of the OptiCell® phantom aligned with HIFU transducer and PCD. Once the experimental set up was ready as shown in Figure 4.3, Ad5, Ad5–PHPMA, or Ad5–AuPEG with SonoVue microbubbles were pumped into the 1-mm flow channel of the OptiCell phantom as described in Section 4.1.3.

The OptiCell® phantom was placed inside a custom-designed holder which was then mounted on a motorized 3-D positioning system (Precision Acoustics, UK). During the experiment, the Opticell® phantom, HIFU transducer, and PCD were immersed in a large tank (110 cm x 60 cm x 70 cm) containing 300 L of degassed and deionized water kept at 37°C using an immersion heater (GD100; Grant Instruments, UK). Since the attenuation in the cell-embedding agarose gel was similar to water [261], no de-rating was performed in reporting pressure amplitudes from calibrations performed in water. The complete block

diagram of the experimental apparatus is shown in **Figure 4.3** while the front view of alignment of the HIFU transducer with the OptiCell® phantom is shown in **Figure 4.4**.

4.1.3 Ultrasound parameters and Ad5 protocol for OptiCell flow phantom

Under different peak rarefactional pressures (PRFP), different types of microbubble oscillations (stable or inertial) can be instigated. Before exposing flowing adenovirus to ultrasound, the ultrasound exposure parameters that yielded regimes of stable and inertial cavitation for SonoVue® inside the OptiCell® flow channel were first identified. Characterisation of these oscillations was achieved by analysing the power spectrum of the passively recorded acoustic emissions as previously described [262]. Acoustic emissions that contain broadband noise indicate unstable nonlinear oscillations and bubble collapse (inertial cavitation) whereas those that evidence ultra-harmonics with minimum broadband noise are associated with stable bubble oscillations over many cycles (stable cavitation) [262]. Quantification of these frequency components was performed in the frequency domain using the Fast Fourier Transform (FFT) of the voltage traces recorded by the PCD. The power spectrum and the contribution of each frequency component to the total energy recorded were determined using a custom made program in Labview 10.0 (National Instruments, UK).

For all exposure regimes, the duty cycles were adjusted at a fixed pulse repetition frequency (PRF) to ensure that the total acoustic energy delivered to the phantom remained constant. In the presence of SonoVue® microbubbles, strong ultraharmonic emissions (stable cavitation) were seen at a PRFP of 180 kPa, 80% duty cycle, and a PRF of 10 Hz; strong broadband emissions (inertial cavitation) were seen at a PRFP 625 kPa, 6.64% duty cycle, PRF 10 Hz and also at PRFP 1250 kPa, 1.66% duty cycle, PRF 10 Hz. As a result, 180 kPa, 625 kPa, and 1250 kPa were chosen as our main *in vitro* testing parameters for the OptiCell flow-channel phantom study for three different adenoviruses (Ad5, Ad5–PHPMA, or Ad5–AuPEG). For these experiments, the total exposure duration was fixed to 10 sec and the total

acoustic energy delivered to the phantom remained constant across exposure regimes. Given that the energy is directly proportional to the square of pressure, when the pressure doubles (e.g. from 625 kPa to 1250 kPa), the duty cycle has to be reduced by a factor of 4 to match the same energy output. These optimal parameters were used for subsequent experiments and data analyses and are referred to only by their PRFP.

These ultrasound parameters were comparable to previous study by Bazan-Peregrino *et al.* which used 360 kPa, 90% duty cycle and PRF of 10 Hz for stable cavitation and 1250 kPa, 6.5% duty cycle and PRF 10 Hz for inertial cavitation [159]. The maximum stable cavitation pressure (180 kPa) obtained from the OptiCell® model was 50% lower than the maximum stable cavitation pressure (360 kPa) reported in Bazan-Peregrino *et al.*'s study; this might be attributable to the use of an OptiCell® unit rather than the customized holder produced by Bazan-Peregrino, and to the fact that the propagation path length to the channel and the channel diameter (1 mm vs. 1.6 mm) were both smaller than in Bazan Peregrino's experiments.

Once the ultrasound parameters had been optimised (see **Table 4.5**), Ad5, Ad5–PHPMA, or Ad5–AuPEG was mixed with ultrasound contrast agent SonoVue® microbubbles (mean diameter of 2.5 µm; Bracco, Italy) in PBS which was flowed through the tumour-mimicking flow-vessel model using a peristaltic pump (Minipulse 2; Gilson, USA) at a flow rate of 1 mL/min. This flow rate corresponds to that of a human malignant tumour in the mass range 1 to 2 g [263]. PBS was degassed for 5 min and then mixed with adenovirus at 1×10^9 v.p./mL and 1×10^7 SonoVue®/mL (20-fold dilution from stock solution). While the adenovirus samples were flowing through, each OptiCell flow channel was targeted at four different spots (15mm apart) with one of the three ultrasound exposure conditions shown in **Table 4.5**. Ad5, Ad5–PHPMA, or Ad5–AuPEG was also flowed through three control channels with no ultrasound exposure at the same concentration, flow rate, and duration. This created a 3 by 4 parameter matrix in which four ultrasound conditions were tested on three

different types of Ad5, making 12 unique OptiCell flow channels for analysis later. This experiment was repeated twice, and all data presented in section 4.2.

Cavitation condition	Stable Cavitation	Inertial Cavitation 1	Inertial Cavitation 2
Frequency	0.5 MHz	0.5 MHz	0.5 MHz
Pressure	180 kPa	625 kPa	1250 kPa
Voltage (mV)	20 mV	70 mV	140 mV
Pulse repetition period	100 ms (10 Hz PRF)	100 ms (10 Hz PRF)	100 ms (10 Hz PRF)
Pulse length	80 ms (40000 cycles)	6.64 ms (3320 cycles)	1.66 ms (830 cycles)
Duty cycle	80%	6.64%	1.66%
Duration	10 sec (100 pulses)	10 sec (100 pulses)	10 sec (100 pulses)

Table 4.5: Ultrasound parameters for OptiCell flow-channel phantom experiment. One stable and two inertial cavitation conditions were carried out inside OptiCell flow-channel while Ad5, Ad5-PHPMA, or Ad5-AuPEG in PBS flew through the channel.

At the end of each experiment, each flow channel was flushed with degassed PBS for 1 min. By using a razor blade, a volume of 10mm length x 3mm width x 2mm height cuboid was extracted directly 2 mm, 4 mm, and 6 mm directly above each flow channel at the ultrasound focal point as shown in **Figure 4.6**. Each cuboid was mixed with 200 μ L of PBS inside a 1.5-mL Eppendorf tube, incubated on a heat box at 70°C for 5 min to allow the agar structure to melt, purified for Ad5 genome content, and quantified for the amount of Ad5 present by qPCR using procedures described in section 3.3.1.

After obtaining qPCR data for the experiments described above, visual evidence of Ad5 infection on the OptiCell model was sought. The same experiment was thus repeated with some modifications. Firstly, the 625-kPa ultrasound condition was not tested as 1250 kPa already provided an inertial cavitation setting, and was more compatible with previous and future *in vivo* experiments. Secondly, Ad5-PHPMA was not used because it could not be reduced by β -mercaptoethanol (BME), so no viral infection could take place since the PHPMA polymer coated Ad5 very well. Thirdly, after each ultrasound exposure condition, half of the OptiCell flow channels were submerged in PBS containing 10 mM BME for 20 min while the other half were submerged in PBS; the presence of BME allowed the cleavage of AuPEG from Ad5 as previously shown in our *in vitro* experiment in section 3.2.1.

Fourthly, all OptiCell phantoms were incubated at 37°C for 24 hr to allow viral infection into the IGROV-1 cells embedded in the agar. Since all Ad5 used in our *in vitro* study carried the GFP reporter gene, once a cell was successfully infected by Ad5, at 24-hr time point, it would express green fluorescent protein.

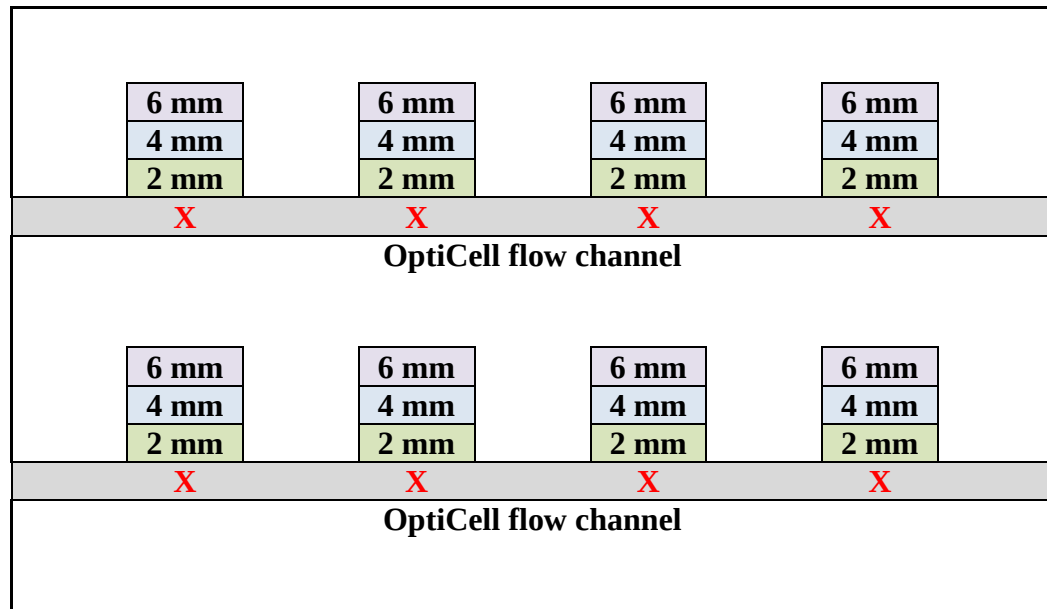


Figure 4.6: Positions of ultrasound exposure per OptiCell channel and sample locations taken for qPCR analysis. Each OptiCell flow channel was exposed to the same ultrasound conditions (180 kPa, 625 kPa, 1250 kPa, or no ultrasound) at four spots (marked with **red X** above) which were 15mm apart; after the exposure, 60-mm³ cuboids 2 mm, 4 mm, and 6 mm above each ultrasound targeting focus were taken out of each OptiCell to quantify the presence of Ad5—an assessment for Ad5 extravasation.

At the 24 hr time point, each OptiCell phantom was imaged under a fluorescence microscope (Eclipse Ti-E; Nikon Instruments Europe, UK), and images of the virally infected cells were taken in a 15 mm x 10 mm plane at 1X magnification (3.2 $\mu\text{m}/\text{pixel}$). FITC filter (excitation at 494 nm and emission at 518 nm) was used to detect GFP expression, and the focus of the microscope targeted at the same ultrasound-targeted four spots per flow channel as shown in **Figure 4.6**. Bright field images of the same locations were also taken. From the images taken, the number of infected cancer cells in each well was counted using the software ImageJ version 1.47 (National Institute of Health, USA).

4.2 *In vitro* experimental results

To better understanding the *in vitro* results obtained from the OptiCell model, this section is divided into three parts: cavitation data of SonoVue® acquired by PCD from the flow channel, qPCR quantification of Ad5 extravasation into the OptiCell model, and the visual evidence of Ad5 infection and extravasation pattern. All statistical analyses in this section were completed using a one-way analysis of variances (ANOVA) followed by a Newman-Keuls test for pairwise comparison of sub-groups.

4.2.1 Results: cavitation data from OptiCell phantom

Time-frequency diagrams of acoustic emissions detected by the PCD provide a useful tool for quantifying and qualifying cavitation activity, with broadband energy corresponding to inertial cavitation and ultraharmonic energy corresponding to stable cavitation [45]. To enable qualification (inertial versus stable cavitation) and quantification (energy recorded) of the type of cavitation activity created by each exposure setting, ultraharmonics of the order $nf_0/2$ and broadband emissions in the range 2 to 15 MHz were extracted and integrated over the exposure duration to create frequency domain power spectrograms (**Figure 4.7**). Acquisition of cavitation signals started 2 sec before the application of HIFU to enable quantification of background electrical noise and continued for 2 sec after HIFU was switched off, so the time range showed in the spectrograms was from 2 to 12 sec for the whole 10-sec duration. In the 180-kPa spectrogram, only ultraharmonics were observed, whereas both 625-kPa and 1250-kPa spectrograms showed sustained broadband noise throughout the exposure.

Extracted from the data from the three power spectrograms, **Figure 4.8** is a single-time point graph showing frequency versus FFT magnitude at time $t = 4$ sec; this served as a better visualisation to show the ultraharmonics from the 180-kPa spectrogram and that only low-integer harmonics remain visible above broadband noise for the 625- and 1250-kPa spectrograms. After integrating the area under the curve in **Figure 4.8** across the entire

exposure duration, the energy of acoustic emissions detected for 625 kPa and 1250 kPa were around 15 and 20 times larger, respectively, than that at 180 kPa; the energy radiated as broadband noise was 30% greater at 1250 kPa compared to 625 kPa.

All spectrograms captured during the experiment were remarkably repeatable ($n = 4$) for the same exposure conditions, suggesting the OptiCell flow channel phantom served as a good controlled environment to provide reproducible cavitation effects at all three pressures. This consistent ultrasound output on each OptiCell flow channel enabled a reliable analysis for the different extravasation effects of Ad5, Ad5-PHPMA, and Ad5-AuPEG later (section 4.2.2).

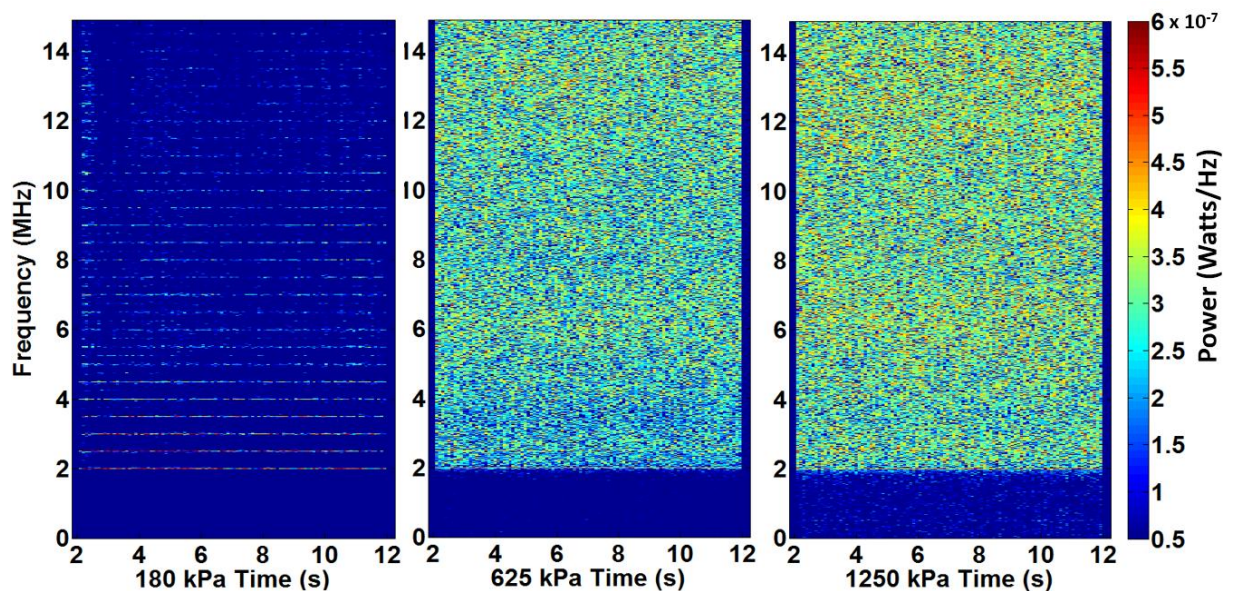


Figure 4.7: Spectrograms of passive cavitation data captured from OptiCell® phantom at various pressures. These spectrograms were obtained by plotting the FFT magnitude (Watts/Hz) over time. The 180-kPa spectrogram is dominated by ultraharmonics of $f_0/2$, while the 625-kPa and 1250-kPa spectrograms indicated that the signal is dominated by broadband noise. The colour scale bar on the right represents relative FFT magnitude.

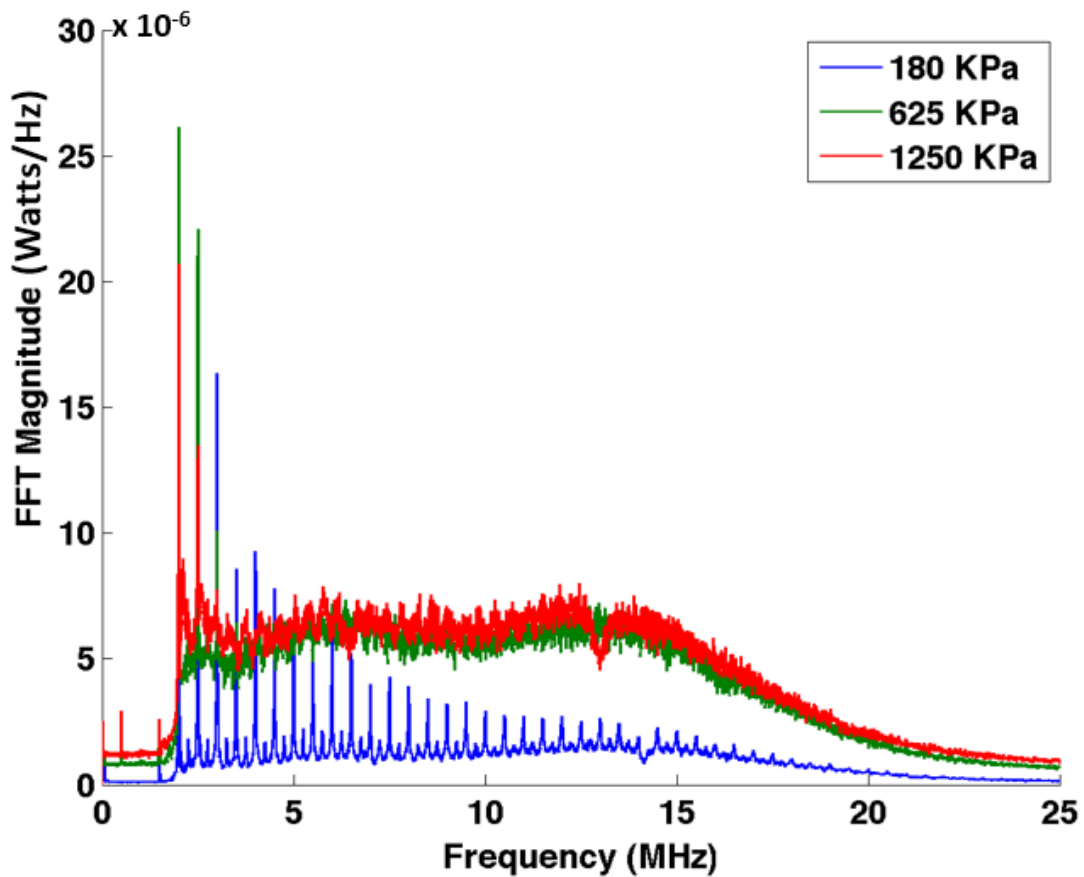


Figure 4.8: A single-time point graph showing frequency versus FFT magnitude (Watts/Hz) at time $t = 4$ sec for different pressures. This is a single-time point snapshot of the three spectrograms shown in Figure 4.7 to allow a better visualisation of ultraharmonic signals for 180 kPa condition and to demonstrate only integer harmonics remain visible for the 625- and 1250-kPa conditions.

4.2.2 Results: adenovirus extravasation in OptiCell flow phantom

As reviewed in Chapter 1, because Ad5 can only infect cells by binding to specific cell membrane receptors, sonoporation is explicitly excluded in this study as a potential drug delivery mechanism and the focus is exclusively on the use of cavitation for extravasation [262]. In addition, the literature section 1.3.1 showed that most cell membrane pore sizes opened under sonoporation were less than 100 nm in size; since a typical Ad5 is 120 nm in size, it is very unlikely that Ad5 has the ability to enter these transient pores opened by sonoporation.

The results of qPCR analysis aimed at quantifying the number of Ad5 viral particles present in the extracted cuboids 2-mm, 4-mm, and 6-mm directly above the ultrasound focus

in the flow channel are shown in **Figure 4.9**. In addition, **Table 4.10** shows a one-way ANOVA (Newman-Keuls multiple comparison) test carried out for all samples (i.e. summing up the total Ad5 cuboids from all distances for the same ultrasound condition and Ad5 modification).

The ‘no ultrasound’ (NUS) result effectively provides the background level in which Ad5, Ad5–PHPMA, and Ad5–AuPEG passively accumulate in the OptiCell phantom, mimicking an *in vitro* EPR effect of these three Ad5 therapeutics; under this control condition, roughly around 2×10^4 copies of Ad5, Ad5–PHPMA, and Ad5–AuPEG were detected within 2-mm from the flow channel, which is less than 0.01% of the originally injected viral dose from the flow channel. Having established a baseline for the NUS groups, there are two ways to analyse the extravasation of Ad5: by varying the pressure for the same Ad5 modification or by varying the type of Ad5 (Ad5, Ad5–PHPMA or Ad5–AuPEG) for the same pressure setting.

In the 180-kPa stable cavitation regime, the amount of Ad5, Ad5–PHPMA, and Ad5–AuPEG detected in the cuboids 2 mm from the vessel increased by a factor of 3, 2, and 11, respectively ($p < 0.05$ for all), relative to no ultrasound (NUS). For the cuboids 4-mm from the vessel, 2-fold ($p > 0.05$), 2-fold ($p > 0.05$), and 8-fold ($p < 0.05$) increases were observed, respectively. For the cuboids 6 mm from the vessel, there was no significant increase in virus accumulation for Ad5 and Ad5–PHPMA ($p > 0.05$), while a 3-fold increase was detected for Ad5–AuPEG ($p < 0.05$), relative to NUS. These results show that stable cavitation of SonoVue® was only significant enough to extravasate Ad5 and Ad5–PHPMA 2-mm away from the flow channel but was powerful enough to drive Ad5–AuPEG at least 6-mm into the tumour-mimicking phantom.

In the 625-kPa inertial cavitation regime, Ad5, Ad5–PHPMA, and Ad5–AuPEG exhibited a 16-fold, 13-fold, and 20-fold ($p < 0.01$ for all) increase of Ad5 accumulation at 2

mm, relative to NUS. A very interesting observation happened at the 4-mm distance in which Ad5 and Ad5–PHPMA increased by a factor of 5 and 6 ($p < 0.05$), respectively, but the much denser Ad5–AuPEG increased by a factor of 83 ($p < 0.001$). Furthermore, even though, both Ad5 and Ad5–PHPMA had no difference ($p > 0.05$) in virus accumulation in the 6-mm cuboids when comparing the NUS and 625-kPa conditions, Ad5–AuPEG still increased by a factor of 25 ($p < 0.001$) 6 mm from the vessel position. These findings demonstrated that the lower end of the inertial cavitation regime at 625-kPa enables the extravasation of Ad5 and Ad5–PHPMA 4 mm away from the flow channel but enables the delivery of Ad5–AuPEG to a distance in excess of 6-mm away from the flow channel. Although all three therapeutics had similar amount (4×10^5) of virus accumulation at the 2 mm distance, Ad5 and Ad5–PHPMA accumulated the most at 2-mm cuboids, whereas Ad5–AuPEG accumulated the most at 4 mm.

The pattern observed in the 1250-kPa inertial cavitation regime versus the control group was very similar to that at 625-kPa with two additional observations: firstly, both Ad5 and Ad5–PHPMA had a 5-fold and 4-fold increase ($p < 0.05$) at 6 mm, showing that the higher level inertial cavitation generated at 1250 kPa was able to drive them more effectively to the 6-mm distance; secondly, for Ad5–AuPEG accumulation at this pressure, the 6-mm distance had the same virus accumulation (around 1×10^6 v.p.) as seen at 2 mm, implying that the actual distance travelled by Ad5–AuPEG was possibly much longer than just 6 mm away from the flow channel (see section 4.2.3 for more visual evidence). Although the 2 mm cuboids for all three Ad5 therapeutics had similar amount of virus accumulation, the 6 mm cuboid of Ad5–AuPEG had two orders of magnitude more Ad5 than the cuboids containing Ad5 and Ad5–PHPMA ($p < 0.001$). The most impressive 324-fold increase in delivery over the control NUS regime was observed at the 4-mm cuboid for Ad5–AuPEG.

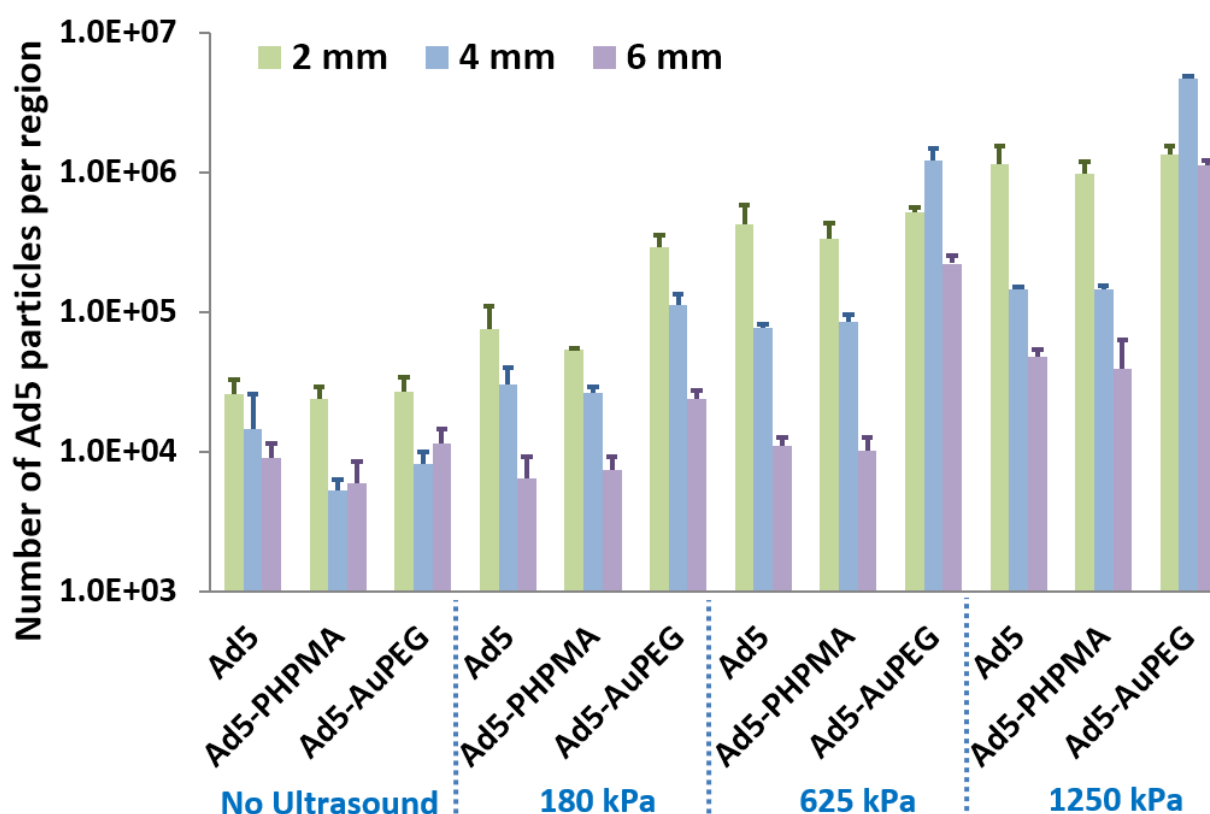


Figure 4.9: Quantification of Ad5 extravasation from the flow channel into the surrounding phantom at different distances away from the channel. qPCR was performed on cell-embedded agar cuboids 2 mm, 4 mm, and 6 mm away from the OptiCell flow channel to quantify the number of Ad5 particles being ‘pushed’ into the tumour-mimicking phantom by no ultrasound or by three different ultrasound pressures (180 kPa for stable cavitation, 625 kPa and 1250 kPa for inertial cavitation). N = 4, mean $\pm$ S.D. shown.

Since all adenovirus therapeutic samples were prepared at the concentration of 1×10^9 v.p./mL, were delivered at 1 mL/min through the flow channel, and exposed to ultrasound targeting for 10 sec per spot, the maximum possible virus accumulation in ultrasound-targeted region is 1.67×10^8 v.p. This allowed us to calculate the percentage of the ‘injected dose’ successfully extravasated in the agar-cell phantom by summing up the total Ad5 detected in all three cuboids per region. Assuming there was an even distribution of v.p. both above and below the flow channels, (as supported by infection studies see below in section 4.2.3) our total Ad5 quantification was multiplied by a factor of 2 to account for that fact that only the cuboids above each flow channel were quantified. For the NUS condition, around 0.04% of Ad5, Ad5-PHPMA, or Ad5-AuPEG would passively accumulate into the OptiCell. In the 180-kPa stable cavitation regime, the accumulation percentages changed to 0.13%, 0.11%, and 0.52% for Ad5, Ad5-PHPMA, and Ad5-AuPEG, respectively. The 625-kPa inertial

cavitation condition yielded 0.62%, 0.53%, and 2.4% of Ad5, Ad5–PHPMA, and Ad5–AuPEG, respectively, in the perivascular space. Last but not least, the 1250-kPa inertial cavitation regime caused accumulation levels of 1.6%, 1.4%, and 8.7% for Ad5, Ad5–PHPMA, and Ad5–AuPEG respectively. However, as previously noted, Ad5–AuPEG at 625 kPa and all three Ad5 therapeutics at 1250 kPa seemed to travel further than the maximum 6-mm distance extracted, so the actual accumulation percentages for these conditions were definitely higher. Interestingly, the conservatively estimated 8.7% accumulation of the 1250kPa-enhanced Ad5–AuPEG in the OptiCell was very similar to the 12% ultimately observed in the *in vivo* tumour model in mice (see Chapter 5).

A second way to analyse **Figure 4.9** is by comparing how the change of Ad5 density affected its extravasation. Recalling the results from section 2.6.5, the densities for Ad5, Ad5–PHPMA, and Ad5–AuPEG were 1.37, 1.33, and 3.35 kg/L, respectively. Even though both Ad5–PHPMA and Ad5–AuPEG increased the volume of Ad5 by roughly 100%, Ad5–PHPMA decreased the effective density by 3% while Ad5–AuPEG increased the effective density by 145%. In the NUS condition, Ad5, Ad5–PHPMA, and Ad5–AuPEG all had equal accumulation of v.p. in the cuboids ($p > 0.05$), suggesting that change in viral density did not affect its passive accumulation into the phantom. In addition, under all inertial and stable cavitation regimes, there was no significant difference between Ad5 and Ad5–PHPMA detected in all cuboids ($p > 0.05$). Interestingly, for all inertial and stable cavitation regimes, the 2.5-fold denser Ad5–AuPEG always extravasated better and accumulated more in the cuboids than either Ad5 or Ad5–PHPMA ($p < 0.001$). For example, comparing the 4-mm distance within the same pressure regime, Ad5–AuPEG had 3.7-fold, 15.6-fold, and 32.4-fold more v.p. accumulation than Ad5 under 180 kPa, 625 kPa, and 1250 kPa, respectively. Two general conclusions could be drawn from these observations: firstly, in the presence of either inertial or stable cavitation, the higher-density Ad5–AuPEG was extravasated significantly further and in larger quantity than either Ad5 or Ad5–PHPMA, which has a similar effective

density to Ad5; secondly, the higher the level of inertial cavitation activity, the greater the enhancement in Ad5–AuPEG delivery compared to Ad5 or Ad5–PHPMA.

Both Ad5–PHPMA (146 nm) and Ad5–AuPEG (149 nm) are almost identical in size and Ad5–AuPEG has an effective density that is 150% higher than either Ad5 or Ad5–PHPMA: this provides compelling evidence that the increase in size produced by the gold conjugation (149nm) compared to the Ad5 original size (117 nm) is not an important factor in cavitation-enhanced extravasation, whilst the increase in Ad5 density clearly plays a significant role.

One Way ANOVA Comparison Test	P < 0.05?	Summary
NUS + Ad5-PHPMA vs 1250 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5-PHPMA vs 625 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5-PHPMA vs 1250 kPa + Ad5	Yes	***
NUS + Ad5-PHPMA vs 1250 kPa + Ad5-	Yes	***
NUS + Ad5-PHPMA vs 625 kPa + Ad5	Yes	***
NUS + Ad5-PHPMA vs 625 kPa + Ad5-PHPMA	Yes	***
NUS + Ad5-PHPMA vs 180 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5-PHPMA vs 180 kPa + Ad5	No	ns
NUS + Ad5-PHPMA vs 180 kPa + Ad5-PHPMA	No	ns
NUS + Ad5-PHPMA vs NUS + Ad5	No	ns
NUS + Ad5-PHPMA vs NUS + Ad5-AuPEG	No	ns
NUS + Ad5-AuPEG vs 1250 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5-AuPEG vs 625 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5-AuPEG vs 1250 kPa + Ad5	Yes	***
NUS + Ad5-AuPEG vs 1250 kPa + Ad5-PHPMA	Yes	***
NUS + Ad5-AuPEG vs 625 kPa + Ad5	Yes	***
NUS + Ad5-AuPEG vs 625 kPa + Ad5-PHPMA	Yes	***
NUS + Ad5-AuPEG vs 180 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5-AuPEG vs 180 kPa + Ad5	No	ns
NUS + Ad5-AuPEG vs 180 kPa + Ad5-PHPMA	No	ns
NUS + Ad5-AuPEG vs NUS + Ad5	No	ns
NUS + Ad5 vs 1250 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5 vs 625 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5 vs 1250 kPa + Ad5	Yes	***
NUS + Ad5 vs 1250 kPa + Ad5-PHPMA	Yes	***
NUS + Ad5 vs 625 kPa + Ad5	Yes	***
NUS + Ad5 vs 625 kPa + Ad5-PHPMA	Yes	***
NUS + Ad5 vs 180 kPa + Ad5-AuPEG	Yes	***
NUS + Ad5 vs 180 kPa + Ad5	No	ns
NUS + Ad5 vs 180 kPa + Ad5-PHPMA	No	ns
180 kPa + Ad5-PHPMA vs 1250 kPa + Ad5-	Yes	***
180 kPa + Ad5-PHPMA vs 625 kPa + Ad5-	Yes	***
180 kPa + Ad5-PHPMA vs 1250 kPa + Ad5	Yes	***
180 kPa + Ad5-PHPMA vs 1250 kPa + Ad5-	Yes	***
180 kPa + Ad5-PHPMA vs 625 kPa + Ad5	Yes	***
180 kPa + Ad5-PHPMA vs 625 kPa + Ad5-	Yes	***

180 kPa + Ad5-PHPMA vs 180 kPa + Ad5-	Yes	***
180 kPa + Ad5-PHPMA vs 180 kPa + Ad5	No	ns
180 kPa + Ad5 vs 1250 kPa + Ad5-AuPEG	Yes	***
180 kPa + Ad5 vs 625 kPa + Ad5-AuPEG	Yes	***
180 kPa + Ad5 vs 1250 kPa + Ad5	Yes	***
180 kPa + Ad5 vs 1250 kPa + Ad5-PHPMA	Yes	***
180 kPa + Ad5 vs 625 kPa + Ad5	Yes	***
180 kPa + Ad5 vs 625 kPa + Ad5-PHPMA	Yes	**
180 kPa + Ad5 vs 180 kPa + Ad5-AuPEG	Yes	***
180 kPa + Ad5-AuPEG vs 1250 kPa + Ad5-	Yes	***
180 kPa + Ad5-AuPEG vs 625 kPa + Ad5-	Yes	***
180 kPa + Ad5-AuPEG vs 1250 kPa + Ad5	Yes	***
180 kPa + Ad5-AuPEG vs 1250 kPa + Ad5-	Yes	***
180 kPa + Ad5-AuPEG vs 625 kPa + Ad5	No	ns
180 kPa + Ad5-AuPEG vs 625 kPa + Ad5-	No	ns
625 kPa + Ad5-PHPMA vs 1250 kPa + Ad5-	Yes	***
625 kPa + Ad5-PHPMA vs 625 kPa + Ad5-	Yes	***
625 kPa + Ad5-PHPMA vs 1250 kPa + Ad5	Yes	***
625 kPa + Ad5-PHPMA vs 1250 kPa + Ad5-	Yes	***
625 kPa + Ad5-PHPMA vs 625 kPa + Ad5	No	ns
625 kPa + Ad5 vs 1250 kPa + Ad5-AuPEG	Yes	***
625 kPa + Ad5 vs 625 kPa + Ad5-AuPEG	Yes	***
625 kPa + Ad5 vs 1250 kPa + Ad5	Yes	***
625 kPa + Ad5 vs 1250 kPa + Ad5-PHPMA	Yes	***
1250 kPa + Ad5-PHPMA vs 1250 kPa + Ad5-	Yes	***
1250 kPa + Ad5-PHPMA vs 625 kPa + Ad5-	Yes	***
1250 kPa + Ad5-PHPMA vs 1250 kPa + Ad5	No	ns
1250 kPa + Ad5 vs 1250 kPa + Ad5-AuPEG	Yes	***
1250 kPa + Ad5 vs 625 kPa + Ad5-AuPEG	Yes	***
625 kPa + Ad5-AuPEG vs 1250 kPa + Ad5-	Yes	***

Table 4.10: One Way ANOVA Newman-Keuls multiple comparison tests for all qPCR results from the OptiCell phantom. All qPCR results of different ultrasound conditions and Ad5 modifications were compared with each other. NUS stands for ‘no ultrasound,’ and a comparison test is statistical significance if $p < 0.05$. There are four outcomes for the ‘summary’ column: ns = no significance; *, **, and *** represent $p < 0.05$, 0.01, and 0.001, respectively.

4.2.3 Results: adenovirus infection in OptiCell flow phantom

The results in the previous section provide evidence of enhanced v.p. accumulation in the perivascular space following cavitation-inducing ultrasound exposure; however, because Ad5 is coated with AuPEG which needs to be cleaved prior to activation of the virus, good accumulation does not necessarily guarantee good infection.

In order to obtain visual evidence of viral extravasation and infectivity under the various ultrasound conditions tested, 12 OptiCell® flow channels were imaged 24 hr after ultrasound exposure and following BME reduction. As described earlier in section 4.1.3, half of these channels were submerged in 10 mM BME for 20 min immediately after the ultrasound exposure, while the other half was submerged in PBS. Both bright field and FITC-filtered images were taken to determine the total cell count and number of virally infected cells (which expressed GFP).

Figures 4.11, 4.12, and 4.13 show the imaging results for no ultrasound, 180 kPa (stable cavitation regime), and 1250 kPa (inertial cavitation regime). All three figures show the bright field images on the top four panels and the FITC-filtered images on the bottom four panels. **Figure 4.11** shows that in the absence of ultrasound but in the presence of SonoVue® microbubbles, Ad5 and Ad5–AuPEG fail to infect any cells simply by passive accumulation, whether under BME reduction conditions or not. **Figure 4.12** demonstrates that under stable cavitation conditions, no Ad5-infected cells could be detected in the presence or absence of a BME reducing environment, whereas Ad5–AuPEG was able to infect a small number of cells immediately surrounding the flow channel in the presence of BME.

Despite extremely high v.p. accumulation evidenced in qPCR data, Ad5–AuPEG infectivity was completely blocked in the absence of BME both in the 180-kPa stable cavitation and 1250-kPa inertial cavitation regimes, supporting the assertion in Chapter 3 that

Ad5 is sufficiently well shielded by AuPEG to ablate its infection. An important conclusion from this observation is that the covalent binding of AuPEG to Ad5 is in no way affected by either ultrasound or inertial cavitation, and can only be cleaved by tumour reducing environment such as the 10 mM BME used in this study.

Figure 4.13 further reveals that in the 1250-kPa inertial cavitation regime Ad5–AuPEG not only showed the highest level of infectivity deeper into the phantom but also exhibited significantly lower infectivity in the region immediately surrounding the flow channel. Compared to Ad5 which showed greatest infectivity nearest the channel, decreasing further away from the channel, Ad5–AuPEG shows little infectivity around the flow channel but extensive infectivity between 2 and 4 mm away from the flow channel. This observation matched our qPCR data showing that under inertial cavitation (for both 625 kPa and 1250 kPa), Ad5–AuPEG accumulations peaked within the 4-mm cuboids, rather than within the 2-mm cuboids as observed for Ad5 and Ad5–PHPMA. These findings have strong clinical implication; studies have shown that deeper tumour regions have higher reducing capacity compared to the tumour periphery which is more adequately vascularised [32]. If Ad5–AuPEG is extravasated deeper into tumour's highly reducing environment by inertial cavitation, AuPEG will be more effectively cleaved and Ad5 will be de-stealthed for reactivation of infection.

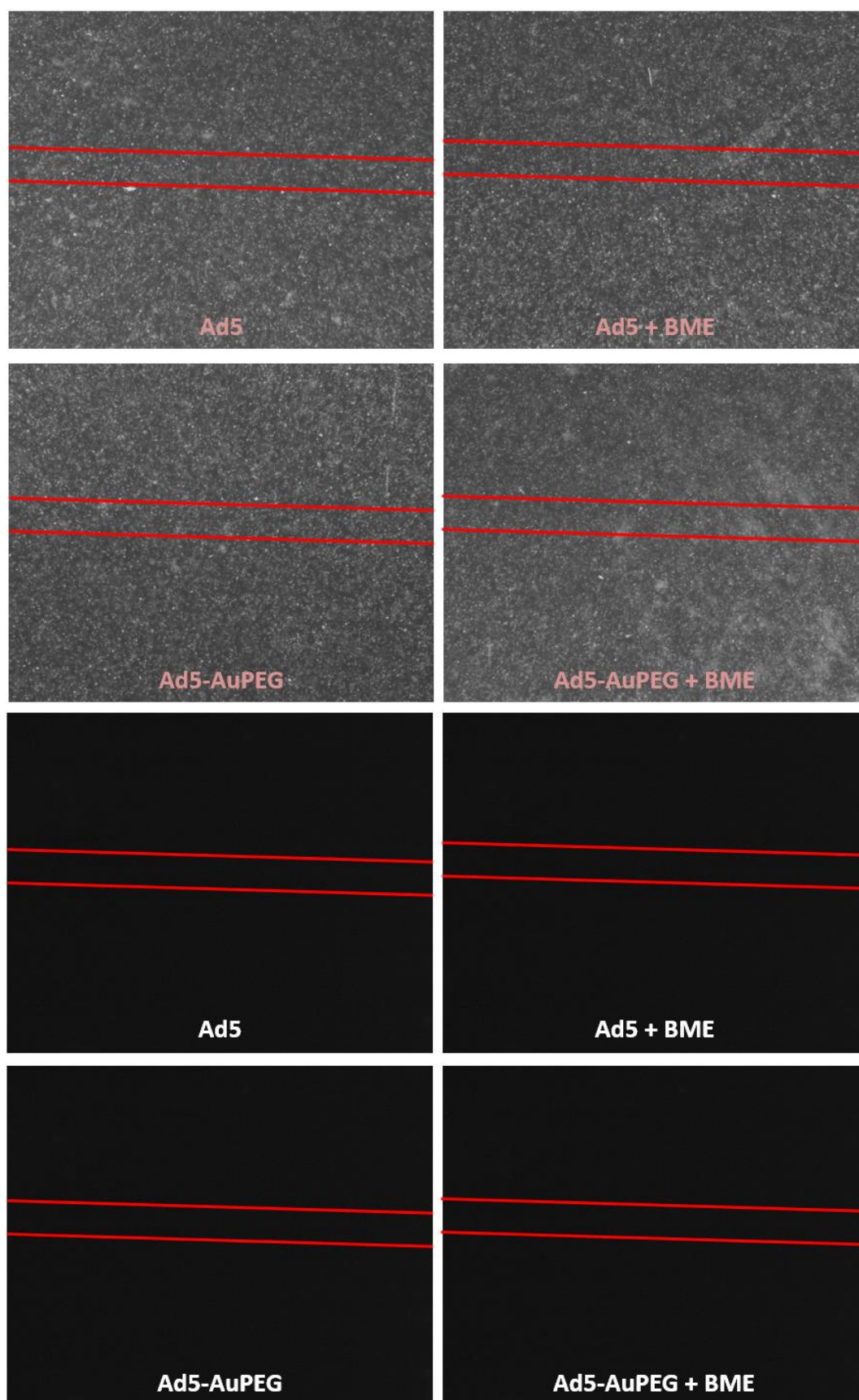


Figure 4.11: Microscopy images (1x magnification) taken of no ultrasound flow channels under bright field and FITC-filter channel. Top four and bottom four images were taken under bright field and FITC-filter channel, respectively. The window in each image is 15 mm length x 10 mm height. The two red lines represent the actual location of the OptiCell® flow channel.

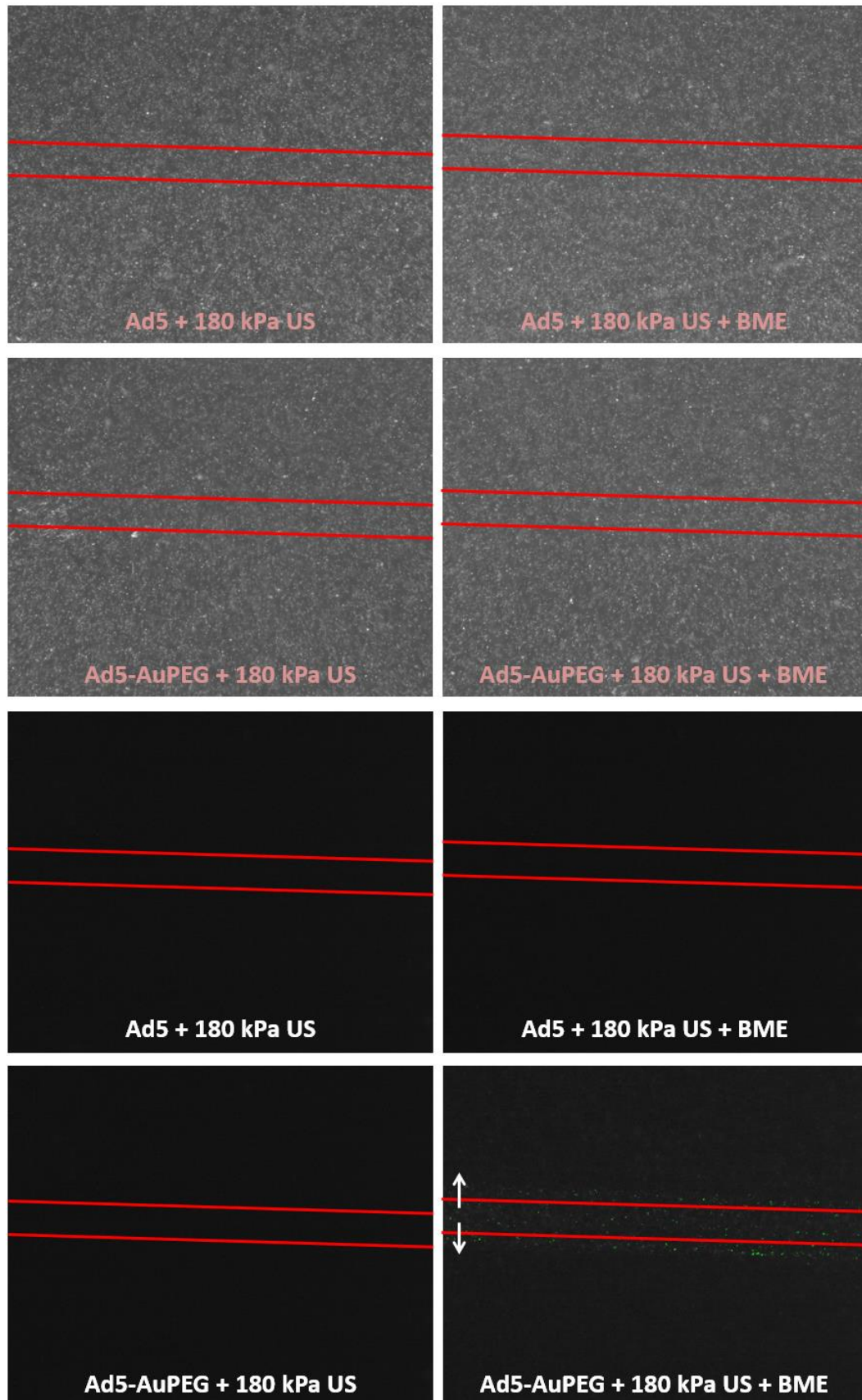


Figure 4.12: Microscopy images (1x magnification) taken of 180-kPa flow channels under bright field and FITC-filter channel. Top four and bottom four images were taken under bright field and FITC-filter channel, respectively. The window in each image is 15 mm length x 10 mm height. The two red lines represent the actual location of the OptiCell® flow channel.

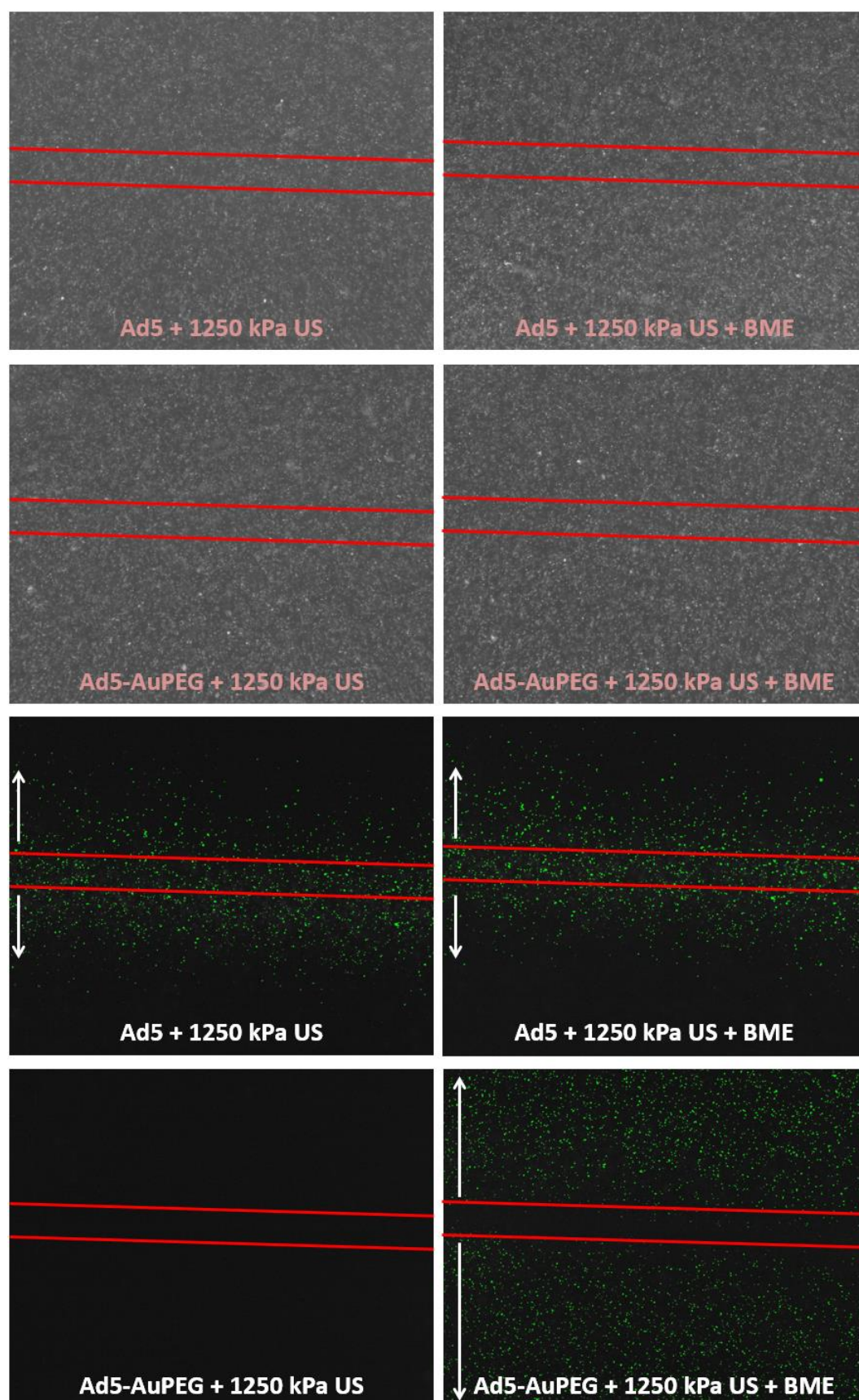


Figure 4.13: Microscopy images (1x magnification) taken at 1250-kPa flow channels under bright field and FITC-filter channel. Top four and bottom four images were taken under bright field and FITC-filter channel, respectively. The window in each image is 15 mm length x 10 mm height. The two red lines represent the actual location of the OptiCell® flow channel.

For all panels displayed in **Figures 4.11 to 4.13**, the total number of bright field cells and GFP-expressed infected cells were counted using the software ImageJ. The total number of detectable cells in all bright field panels was consistently around $12,000 \pm 250$ cells (no significant difference from each other; $p > 0.05$). The total number of GFP-expressing IGROV-1 cells for each condition is shown in **Figure 4.14**. By combining the cell count of bright field images with the GFP-expressing images, several important conclusions can be drawn. Firstly, because a fairly uniform cell count was recorded across all images under bright field, it could be concluded that the cell concentrations in all OptiCell® phantoms were consistent. Secondly, even though only Ad5–AuPEG + BME were able to infect 126 cells on average under the 180-kPa stable cavitation condition, this represented only 1% of the cell population being infected but was still significantly different from the three other groups (Ad5, Ad5 + US, and Ad5–AuPEG) with no viral infection at all ($p < 0.001$). Thirdly, under the 1250-kPa inertial cavitation regime, both Ad5 and Ad5 + BME were able to infect around 2100 (18%) of the total cell present, and BME has no influence on un-modified Ad5 infectivity ($p > 0.05$). Fourthly, Ad5–AuPEG + BME under the same inertial cavitation regime resulted in some 3470 cells (29%) being infected; this was not only significantly higher ($p < 0.01$) than its Ad5 counterparts but also along the same line as the maximum 42% infection that was observed in our 96-well plate *in vitro* results reported in section 3.2.1. Finally, since the microscope could only focus on and image one layer (approximately 50 μm in thickness) of the 3-mm thick OptiCell® phantom when these images were taken, an absolute total cell count or infected cell per region was not precisely possible; as a result, it was best to analyse these data from percentage point of view instead of absolute number. Taking several images at various depths at the same region and adding them up was not an ideal option because the fluorescence of these GFP-expressed cells could easily bleach through several layers. In addition, the OptiCell® phantom is not transparent, so while GFP-expressing cells at different layers could be detected using the FITC channel, light in bright

field could only penetrate passing the first few layers of OptiCell®. These obstacles suggest that, if the ‘stacking’ method was implemented to calculate the absolute number, the total number of the infected cells would be over-estimated.

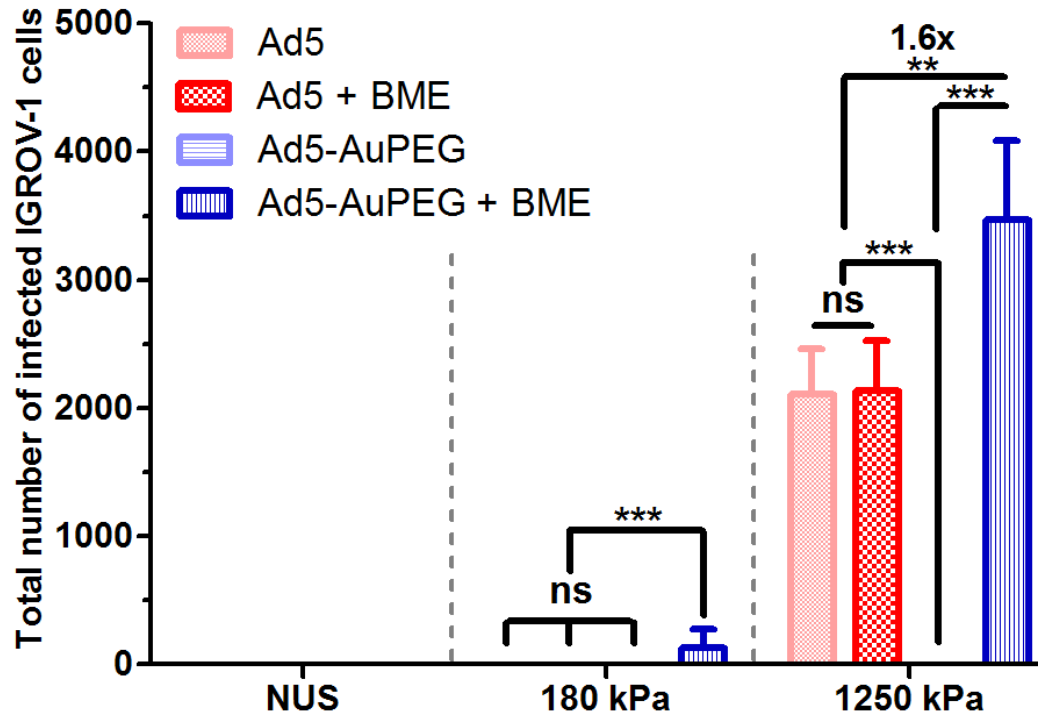


Figure 4.14: Total number of virally infected IGROV-1 cells for each ultrasound and reducing environment condition. The no ultrasound (NUS) region showed no infected cells, while stable (180 kPa) and inertial (1250 kPa) cavitation regions were able to detect GFP-expressed infected cells. N = 4, standard deviations shown; ns = no significance; ** and *** represent $p < 0.01$ and 0.001 , respectively.

Last but not least, the distances between the furthest infected IGROV-1 cells and the centre of the flow channel for each sample were measured, and the results are shown in **Figure 4.15**. Unlike the previous cell count figures where the four images ($n = 4$) taken per sample were solely relied upon, the distances measured for this figure came from eight data points per sample ($n = 8$) since we were able to measure the extravasation distance both above and below each ultrasound target spot on the flow channel. In the stable cavitation regime, no Ad5 was diffused out of the flow channel while Ad5–AuPEG was able to infect cells 0.56 mm on average away from the flow channel. Under the inertial cavitation regime, Ad5 was able to infect 3.5 mm on average away from the flow channel, while Ad5–AuPEG infected cells at an average of 7.9 mm away from the flow channel. Since each microscope image was

only able to capture ± 5 mm away from the flow channel, the total cell count for virally infected GPF-expressed IGROV-1 cells shown in **Figure 4.14** was an underestimation for Ad5–AuPEG under the 1250 kPa pressure. The denser Ad5–AuPEG travelled 2.3 times ($p < 0.001$) further than Ad5 alone under inertial cavitation, again validating the hypothesis that denser Ad5 has the tendency to extravasate deeper into tumour-mimicking tissue due to improved momentum transfer from inertially cavitating SonoVue® microbubbles onto the viral particles.

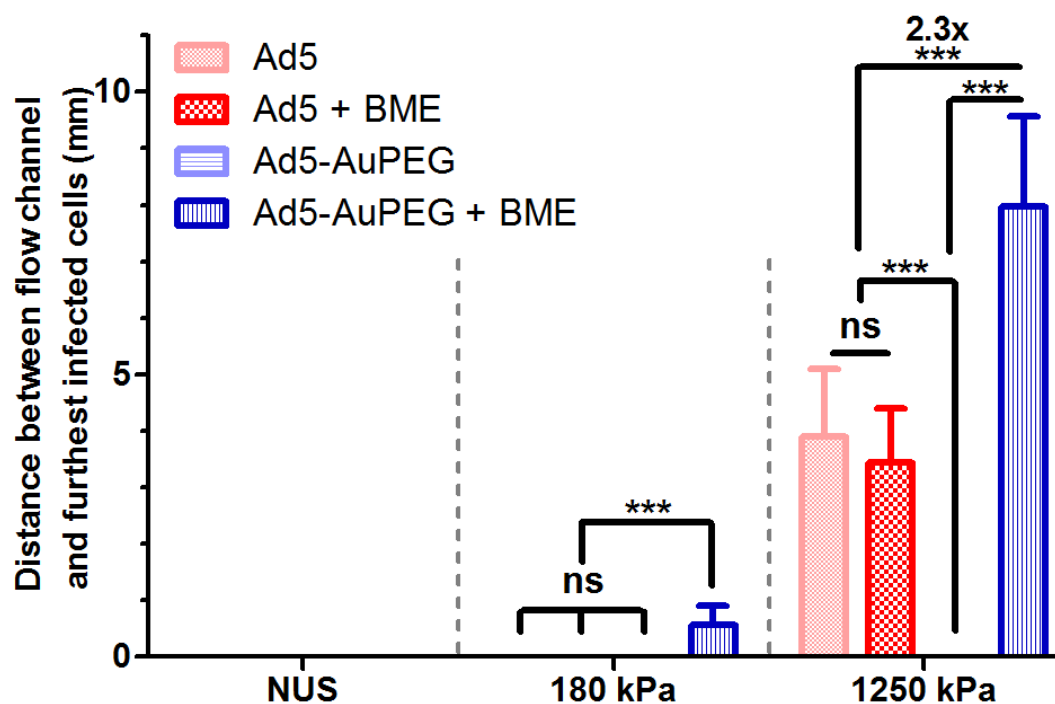


Figure 4.15: Average distances between OptiCell flow channel and furthest virally infected cells. The no ultrasound (NUS) region showed no infected cells, while stable (180 kPa) and inertial (1250 kPa) cavitation regions showed some adenovirus infection deeper into the phantom. $N = 8$, S.D. shown; ns = no significance; *** indicates $p < 0.001$.

4.3 Summary

This chapter demonstrated, for the first time, that under either stable or inertial cavitation conditions, Ad5–AuPEG significantly outperformed Ad5 in terms of extravasation and infection in the tumour-mimicking flow-channel environment. Increasing the density of Ad5 by 145% by creating Ad5–AuPEG makes it possible to enhance the extravasation of v.p. by more than 30-fold in the 1250-kPa inertial cavitation regime. Furthermore, the greater the

ultrasound pressure, the higher the extravasation and infection ratios of Ad5–AuPEG relative to Ad5.

To explain this observation and strong correlation, it is hypothesised that when two particles with similar size have reached their terminal velocities in a given environment, the denser particle yields a higher momentum (the product of mass and velocity) since its mass is larger. During inertial cavitation, the collapsing of microbubbles produces microstreaming and radiation force, which were then immediately transfer to Ad5, Ad5–PHPMA, and Ad5–AuPEG. Given that the pressure created in the OptiCell® cancer cell-embedded agar environment was very high, Ad5, Ad5–PHPMA, and Ad5–AuPEG reached their terminal velocities instantly followed the destruction of SonoVue®. Since Ad5 and Ad5–PHPMA have similar density of 1.37 and 1.33 kg/L, respectively, their resulting momenta were similar and thus travelled into the same depth away from the flow channel. On the other hand, since the density of Ad5–AuPEG is 3.35 kg/L, it created a momentum that is 145% greater than that of either Ad5 or Ad5–PHPMA and helps Ad5–AuPEG travel deeper into the agar phantom (Chapter 4) and solid tumours (Chapter 5).

The first thing that is going on is that I have two particles of the same size but different densities. The only way for the particles to travel further (and thus higher momentum) is that they are both reaching terminal velocity. At terminal velocity, the denser the particle, the heavier it is. Momentum = mass x velocity. To reach the terminal velocity, the collapsing of microbubbles under inertial cavitation produces radiation forces and microstreaming which transmit to Ad or Ad-AuPEG.

Unlike the adenovirus used by Bazan-Peregrino *et al.* [159], all adenoviruses used in the work presented in this chapter were non-oncolytic; as a result, viral replication was not studied in the days following initial ultrasound exposure. However, this limitation will be addressed in our *in vivo* experiments described in Chapter 5.

The OptiCell tumour-mimicking flow-channel model presented in this chapter may offer many features for testing extravasation of drugs into a tissue-mimicking material not only for ultrasound-enhanced delivery but also for diffusion, convection, and spreading of other drugs or nano-medicines. The base area of OptiCell® holder is exactly the same as the area of a typical 96-well plate. This allows immediate imaging following experiments involving virus or therapeutics carrying a reporter gene or fluorescence tags to test for passive or active targeting to virtually any type of cell (cancerous or non-cancerous) that can be embedded within agar and tissue culture medium.

Finally, if increasing effective particle density does indeed result in enhanced cavitation-mediated extravasation, the underlying principle is readily extendable to almost any application of ultrasound-enhanced drug delivery. The AuPEG chemistry can be applied to almost any free or encapsulated therapeutic whilst it may also be possible to substitute Au for other types of less expensive but also heavier metal nanoparticles or quantum dots. In addition, the extent and depth of this delivery can be adjusted by controlling the mode and extent of cavitation. This may be especially useful in transdermal immunisation where vaccines need to be deposited in specific immune-favourable skin layers.

CHAPTER 5

***IN VIVO* STUDY OF PEGYLATED GOLD- COATED ADENOVIRUS WITH ULTRASOUND**

5. *In vivo* study of PEGylated gold-coated adenovirus with ultrasound

The previous chapters of this thesis have focussed solely on the *in vitro* testing of Ad5–AuPEG constructs and compared the results with those obtained with Ad5, Ad5–PEG, and Ad5–PHPMA. In this chapter, the *in vivo* characteristics of these Ad5-modified therapeutics were studied. Plasma circulation half-life and tissue bio-distribution were first characterised; later the best candidates were investigated further. In particular, they were combined with ultrasound targeting to test cavitation assisted uptake into tumours and the impact such uptake may have on anti-tumour efficacy. Lastly, a complete survival study on selected optimal candidates was conducted to test the efficiency of Ad5–AuPEG in the presence and absence of ultrasound-enhanced cavitation within tumours.

The aims of this chapter were to:

- 1) Discuss two common mouse models used in *in vivo* studies of virotherapy.
- 2) Create a profile of plasma circulation and bio-distribution for each of Ad5, Ad5–PEG, Ad5–PHPMA, and Ad5–AuPEG in the absence of ultrasound.
- 3) Determine the best Ad5 therapeutic to be used for ultrasound-enhanced virotherapy.
- 4) Create a bio-distribution profile for ultrasound-enhanced virotherapy with and without the coating of AuPEG on Ad5.
- 5) Characterise the inertial cavitation activity achieved after co-injection of Ad5 therapeutics with SonoVue® microbubbles.
- 6) Study the long-term efficacy effects of ultrasound-enhanced virotherapy with and without the coating of AuPEG on Ad5.

5.1 Mouse models for testing Ad5

Two mouse models were used in this study: BALB/c and nu/nu CD1 female mice. BALB/c is a commonly used mouse model for *in vivo* experiments where murine cancer cells are implanted (syngeneic), while CD1 is widely used for the implantation of human cancer cells (xenograft). Wild type mouse strains will reject cancer cells from different species; however, CD1 mice carry a deteriorated or absent thymus, resulting in a much lower production of T cells and an inhibited immune system [264]. As a result, CD1 mice are most receptive to tissue and tumour grafts from different species. The homozygous genotype nu/nu is used to describe the phenotype of CD1 mouse without any body hair, making them ‘nude.’ Female mice were preferred because of their less aggressive nature.

In any *in vivo* study design, if deemed appropriate, the BALB/c mouse model should be used instead of the CD1 mouse model because the former yields a better and competent immune system that enables the subject to stay healthy for longer, and allows isografts/allografts to grow faster than xenografts grow on CD1s. However, for studies involving oncolytic Ad5, a human cell line must be used as murine cell lines only permit an aborted Ad5 replication cycle to take place. For these reasons, uptake studies were performed in BALB/C with CT26 tumours whilst infection/survival studies were performed in CD1 with HepG2 tumours.

Even though mice are the most convenient and commonly used animal model before human clinical trials can be conducted, there are a few limitations that lead to a failure of murine models to predict the behaviour of Ad5 delivery in humans. As already discussed, a study by Carlisle *et al.* has demonstrated that human, but not murine, erythrocytes may have a function in preventing systemic Ad5 infection by acting as circulating ‘virus traps’ [250]. Carlisle *et al.* raised the crucial question as to whether using murine models for intravenous Ad5 therapeutics is a good predictor of the actual pharmacokinetic behaviour of systemic Ad5

in humans. The study showed that more than 90% of the Ad5 bound to human erythrocytes after only 30 min of interaction, whilst the same Ad5 incubation with murine blood resulted in less than 10% of the total dose binding to erythrocytes [250]. Secondly, the sizes of endothelial fenestrae in liver sinusoids (highly specialised capillaries) for human and mouse are 107 ± 1.5 nm and 141 ± 5.4 nm, respectively [253]; the diameter of a typical Ad5 is 120 nm, meaning that it can passively extravasate more effectively into mouse liver whilst may limit liver capture in humans. To reduce liver capture in mice, clodronate is usually intravenously administrated to suppress Kupffer cells (KC; which sequester Ad5) in mouse liver prior to the delivery of the Ad5. This approach provides better plasma circulation [97]; however, pre-administration of clodronate might not be necessary or appropriate for clinical trial of testing Ad5 [265]. Thirdly, the pre-immune status of humans is also dramatically different to that of mice which are naïve to the exposure of Ad5. Such pre-immunity will have a dramatic impact on the clearance pathways and infection efficiencies in humans [58, 266]. Fourthly, a study by Parker *et al.* has demonstrated that although hepatocyte transduction by Ad5 is mediated by hexon-coagulation FX interaction, human sera can actually efficiently block FX-mediated cellular binding and thus prevent the transduction of Ad5 *in vitro*; in contrast, in mice, sera does not block such interaction [266]. Fifthly, Ad5 actually lacks the ability to replicate in any mouse cells; hence, any virotherapy strategy will look more impressive in mouse xenograft models than other animal models [267]. A more permissive model would Syrian hamsters, which can truly test the selectivity of Ad5 before proceeding on to clinical trial [267].

In summary, the combination of blood cell interactions, size restrictions, and antibody binding prevents Ad5 access to and infection of hepatocytes in humans. These mechanisms are not applicable to mice which, perhaps coincidentally, exhibit high levels of hepatic infection and toxicity when exposed to Ad5.

5.2 *In vivo* testing of Ad5 therapeutics for pharmacokinetics and bio-distribution

Before answering the question of whether the density of Ad5 plays an important role in ultrasound-enhanced virotherapy *in vivo*, the pharmacokinetics and bio-distribution of the Ad5-modified therapeutics were studied. It has been shown that the impact of ultrasound on tumour accumulation is more reliable and effective when circulation of Ad5 is extended [261]. Since this was a short-term study on plasma circulation and bio-distribution, BALB/c female mice were used and subcutaneously implanted with CT26 cells (mouse colonic carcinoma). This model was chosen to allow experiments to be performed in fully immune competent mice bearing tumours with a physiologically relevant vascularisation. All animal experiments in this study were performed in accordance with the terms of UK Home Office guidelines and the UK Coordinating Committee on Cancer Research's *The Guidelines for Welfare of Animals in Experimental Neoplasia*.

5.2.1 Experimental methods and mouse model

32 five-week-old BALB/c female mice were obtained from the Biomedical Services Unit from the John Radcliffe Hospital (Oxford, UK). CT26 cells were maintained in tissue culture flasks with DMEM cell culture media (10% FBS) using the cell culturing procedures described in section 3.2. At least 1×10^{11} copies of Ad5, Ad5-PEG, Ad5-PHPMA, or Ad5-AuPEG were synthesised and purified, as described in sections 2.3 and 2.4. Before *in vivo* administration, DLS measurements and ELISA were performed to ensure the increased sizes and coatings of the Ad5-modified therapeutics were of a good and comparable quality to the previously reported data in Chapters 2 and 3.

To establish a CT26 tumour, each mouse was subcutaneously implanted with 100 μ L of DMEM containing 5×10^5 CT26 cancer cells into the flank. Once the tumours reached a size between 100 and 150 mm³, 16 mice were intravenously (i.v.) pre-administrated with 150 μ L clodronate liposomes (Foundation Clodronate Liposomes, Netherlands) to sequester KCs.

24 hr later, the 16 clodronate pre-injected mice and the 16 non-clodronate injected mice were randomly divided into eight groups of four mice ($n = 4$), and each group was i.v. administrated with 1×10^{10} Ad5, Ad5-PEG, Ad5-PHPMA, or Ad5-AuPEG per mouse using a 29-gauge insulin syringe (Becton Dickinson, UK). 20- μ L blood samples were taken from the tail vein at 5, 15, and 30 min after each Ad5 therapeutic injection, diluted to 200 μ L in PBS, and stored at -20°C immediately. Tumour and liver samples were extracted following cull at 35 min and homogenised using an Ultra-Turrax homogenizer (model T18; IKA Laboratory Equipment, Germany) in PBS to a concentration of 125 mg/mL. DNA was extracted from blood and homogenised tissue samples using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, UK), as previously described in section 3.3.1. qPCR was used to detect the presence of Ad5 DNA in all extracted DNA samples, as previously described in section 3.3.1.

All statistical analyses in this chapter were completed using a one-way analysis of variances (ANOVA) followed by a Newman-Keuls test for pairwise comparison of subgroups. All data are typical of two or three repeat experiments (except the survival study which was conducted only once). All data are reported such that *, **, and *** represent p-values < 0.05 , 0.01 , and 0.001 , respectively.

5.2.2 Results: plasma circulation profile

In vivo studies were performed with or without pre-administration of clodronate liposomes (which results in depletion of KCs and lower liver capture of Ad5) [268]. After i.v. administration of Ad5, Ad5-PEG, Ad5-PHPMA or Ad5-AuPEG, blood samples were taken at 5, 15, and 30 min, and tumour and liver samples were extracted following cull at 35 min. Blood circulation profiles of Ad5, Ad5-PEG, Ad5-PHPMA and Ad5-AuPEG are shown in **Figure 5.1**.

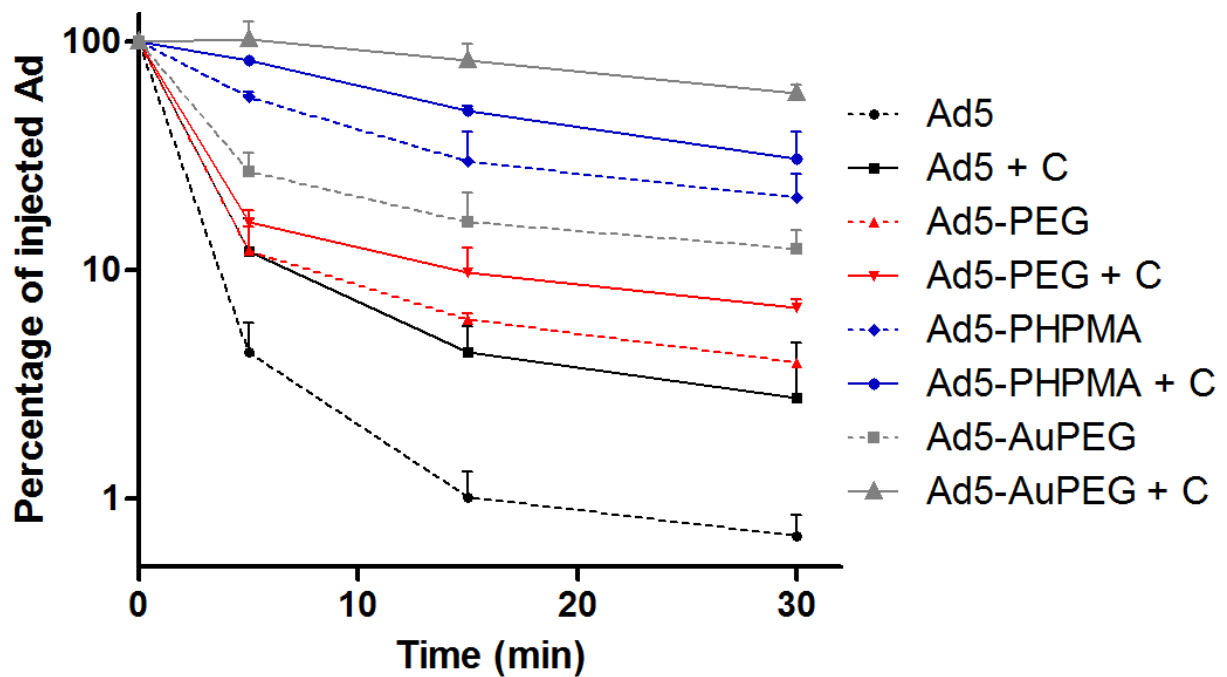


Figure 5.1: In vivo circulation assessment of injected Ad5, Ad5-PEG, Ad5-PHPMA, and Ad5-AuPEG. 1×10^{10} copies of sample were injected IV into BALB/c mice bearing CT26 tumors and blood sampling and quantification performed by qPCR. “C” (solid line) represents administration of clodronate liposomes 24 hr before sample injection, whereas dash lines represent non-clodronate-treated mice. Each group had four mice ($n = 4$), S.D. shown.

The control Ad5, Ad5-PEG, and Ad5-PHPMA circulation data, with or without clodronate, was comparable to previously published results [88, 97, 113]. 30 min after Ad5 administration, 0.7% and 2.8% of Ad5 were detected in blood plasma of control mice and clodronate-treated mice, respectively (**Figure 5.1**); Green *et al.* reported very similar findings of 0.8% and 2.5%, respectively [97], and Gao *et al.* also reported that 1% of Ad5 remained in non-clodronate-treated mouse plasma 30 min after i.v. administration [113]. Together, these results showed the depletion of KCs by clodronate treatment was able to increase the quantity of un-modified Ad5 circulating at 30 min 3 to 4-fold.

The percentages of Ad5-PEG remaining at 30 min were 3.9% and 6.8% for non-clodronate mice and clodronate-treated mice, respectively (**Figure 5.1**). Gao *et al.* reported a similar level of 4% plasma Ad5-PEG detected at 30 min in non-clodronate-treated mice [113], and Alemany *et al.* calculated that around 10% of Ad5-PEG were still circulating in the plasma of clodronate-treated mice [88]. These findings showed that the clodronate treatment enhanced Ad5-PEG plasma circulation profile by a factor of 2 at 30 min.

Also at the 30-min time point, 21% and 31% of Ad5–PHPMA were recovered from blood plasma of mice without clodronate treatment and mice with clodronate treatment, respectively (**Figure 5.1**); although Green *et al.* showed a much higher Ad5–PHPMA percentages of 35% and 90%, respectively, the authors had optimised the Ad5–PHPMA circulation by pre-dosing the mice with 10^{11} Ad5–PHPMA prior to the ‘actual’ Ad5–PHPMA administration and by delivering their Ad5–PHPMA at a ten times higher dose than the dose used in this DPhil study. The authors also discussed that without the pre-dosing treatment and higher dose injection, they would expect a much lower Ad5–PHPMA circulating in the plasma at 30 min [97]. These findings therefore provide useful information on the saturation levels of these clearance mechanisms in mice. Another earlier paper by Green *et al.* demonstrated that the 30-min plasma circulation of Ad5–PHPMA was 12% without any pre-dosing treatment and clodronate pre-administration; however, the paper did not study the effects of clodronate treatment on mice for enhancing Ad5–PHPMA circulation [118]. These results showed clodronate treatment was able to improve the 30-min circulation profile of Ad5–PHPMA by a factor of 1.5 to 3. However, the i.v. injection of clodronate liposomes cannot be extrapolated to clinical trials because several long-term safety issues needed to be solved first, and oral administration of clodronate liposome is not yet available [269].

The percentages of Ad5–AuPEG remaining at 30 min were 12% and 59% for control mice and clodronate-treated mice, respectively (**Figure 5.1**). Since this was the first time a virus-gold therapeutic was tested *in vivo* for circulation, no other published literature was found for a direct comparison. The 5-fold increase of Ad5–AuPEG in clodronate-treated mice suggested that KC capture of Ad5–AuPEG was a key to its clearance. In clodronate pre-treated mice, the half-life of Ad5–AuPEG was more than 30 min, meaning it outperformed all other groups, including Ad5 ($t_{1/2} = 2$ min), Ad5–PEG ($t_{1/2} = 4$ min), and Ad5–PHPMA ($t_{1/2} = 18$ min).

While the clodronate treatment was able to increase the 30-min plasma circulation percentages of Ad5-PEG and Ad5-PHPMA by a factor of 2-3, it provided a 5-fold increase in the percentage of circulating Ad5-AuPEG at 30 min. Notably, in the absence of clodronate liposome pre-treatment, Ad5-PHPMA showed extended circulation compared to Ad5-AuPEG, suggesting KC capture of Ad5-AuPEG is a more important clearance mechanism than for Ad5-PHPMA or Ad5-PEG. A possible explanation for this is that solid gold nanoparticles, despite being PEGylated, are more susceptible to detection by macrophages than soft biocompatible materials such as PHPMA and PEG. Further optimization of the Au:PEG ratio may help reduce such KC mediated capture. The absence of pre-existing immunity and erythrocyte CAR and CR1 makes extrapolation of mouse pharmacokinetics to humans difficult. Furthermore, each complete circulation takes approximately 1 min in humans compared to 5 sec in mice. However, in humans, it is likely that before Ad5 vectors reach the liver, they will be sequestered by erythrocytes [250] and/or leukocytes [270]. Capturing by KC complement receptors will then mediate clearance [271]. As Ad5-AuPEG has very effective coating and low antibody binding (see sections 2.6 and 3.1), it may be predicted that this erythrocyte-KC mechanism is replaced by a more direct Ad5-AuPEG-KC interaction caused by the gold.

A one-way ANOVA Newman-Keuls comparison test was performed for the 30 min time point data, and the results are shown in **Table 5.2**; the letter ‘C’ stands for with clodronate-treated mice, and *, **, and *** represent $p < 0.05$, 0.01, and 0.001, respectively.

One Way ANOVA Newman-Keuls Comparison Test	P < 0.05?	Summary
Ad5 vs Ad5-AuPEG + C	Yes	***
Ad5 vs Ad5-PHPMA + C	Yes	***
Ad5 vs Ad5-PHPMA	Yes	***
Ad5 vs Ad5-AuPEG	Yes	*
Ad5 vs Ad5-PEG + C	Yes	**
Ad5 vs Ad5-PEG	Yes	*
Ad5 vs Ad5 + C	Yes	*
Ad5 + C vs Ad5-AuPEG + C	Yes	***
Ad5 + C vs Ad5-PHPMA + C	Yes	***

Ad5 + C vs Ad5-PHPMA	Yes	***
Ad5 + C vs Ad5-AuPEG	Yes	***
Ad5 + C vs Ad5-PEG + C	Yes	*
Ad5 + C vs Ad5-PEG	No	ns
Ad5-PEG vs Ad5-AuPEG + C	Yes	***
Ad5-PEG vs Ad5-PHPMA + C	Yes	***
Ad5-PEG vs Ad5-PHPMA	Yes	***
Ad5-PEG vs Ad5-AuPEG	Yes	*
Ad5-PEG vs Ad5-PEG + C	Yes	*
Ad5-PEG + C vs Ad5-AuPEG + C	Yes	***
Ad5-PEG + C vs Ad5-PHPMA + C	Yes	***
Ad5-PEG + C vs Ad5-PHPMA	Yes	**
Ad5-PEG + C vs Ad5-AuPEG	No	ns
Ad5-AuPEG vs Ad5-AuPEG + C	Yes	***
Ad5-AuPEG vs Ad5-PHPMA + C	Yes	***
Ad5-AuPEG vs Ad5-PHPMA	Yes	*
Ad5-PHPMA vs Ad5-AuPEG + C	Yes	***
Ad5-PHPMA vs Ad5-PHPMA + C	Yes	**
Ad5-PHPMA + C vs Ad5-AuPEG + C	Yes	***

Table 5.2: One Way ANOVA Newman-Keuls multiple comparison tests for Ad5 therapeutics in the presence and absence of clodronate (C) treatment. 30-min plasma percentages of different Ad5 modifications, with and without clodronate ‘C’ treatment, were compared with each other. The ANOVA comparison test is statistical significance if $p < 0.05$. There are four outcomes for the ‘summary’ column: ns = no significance; *, **, and *** represent $p < 0.05$, 0.01, and 0.001, respectively.

All modified and un-modified Ad5 samples achieved a significantly longer circulation time when clodronate liposomes were pre-administered into the mice ($p < 0.05$). This was important when it came to experimental design of the subsequent survival study. Ad5–AuPEG + C showed significantly improved circulation in mice than any other Ad5 therapeutics ($p < 0.001$). However, Ad5–AuPEG alone without the pre-administration of clodronate had significantly shorter circulation time than both Ad5–PHPMA ($p < 0.05$) or Ad5–PHPMA + C ($p < 0.001$).

For the first time in over a decade of development, this study enables a direct comparison of the blood circulation of Ad5–PEG and Ad5–PHPMA to be made and confirms the hypothesis that improved stealthing with PHPMA improves circulation kinetics compared to conventional PEGylation ($p < 0.001$) in both control mice and clodronate-treated mice.

Notably, in the presence of clodronate liposome pre-treatment, Ad5–AuPEG showed extended kinetics compared to Ad5–PEG or Ad5–PHPMA, indicating that the superior stealthing achieved with Ad5–AuPEG versus Ad5–PEG or Ad5–PHPMA, as demonstrated *in vitro* by ELISA shown in section 3.1, impacted directly on circulation and hepatic capture *in vivo*. Crucially, TNBS analysis showed this improved stealthing with Ad5–AuPEG was achieved with modification of just 6% capsid amine groups compared to 74% with Ad5–PHPMA or 56% with Ad5–PEG (**Table 2.8**). Achieving effective stealthing with minimal surface modification is an important goal; once within tumours, reactivation of Ad5 infection is required and maintenance of the capsid surface integrity is needed for infection. Hence, the small percentage of surface modification with Ad5–AuPEG was crucial for the subsequent survival study, where the tumour reducing micro-environment was needed for effective cleavage of the disulphide bonds between AuPEG and Ad5 for reactivation of infection.

5.2.3 Results: tumour and liver bio-distribution data

After the 30-min blood samples were taken, all mice were culled at 35 min to enable extraction of livers and tumours. After tissue homogenisation, DNA purification, and qPCR were conducted, the total Ad5 detected in the liver and tumour were graphed as percentage of the original injected dose (10^{10} copies) in **Figures 5.3** and **5.4**.

With or without clodronate liposomes, more than 90% of Ad5 and Ad5–PEG was captured by the liver, and they were not different from each other ($p > 0.05$). This indicates that, in accordance with previous studies and the circulation data, Ad5 and Ad5–PEG are captured very effectively by the liver. Both of the hepatic clearance mechanisms (passage through sinusoidal endothelial fenestrae and KC capture) can clear Ad5 and Ad5–PEG, so removal of KC does not dramatically alter liver capture. In the absence of clodronate liposomes, similar-levels, 66% of Ad5–PHPMA and 68% Ad5–AuPEG, were captured by the liver. Upon the administration of clodronate liposomes, liver capture of Ad5–PHPMA and

Ad5–AuPEG significantly decreased to 48% ($p < 0.05$) and 21% ($p < 0.001$), respectively. This is in accordance with the plasma circulation data from **Figure 5.1**, and demonstrates that clodronate-treated mice showed significantly improved circulation and decreased hepatic capture for both Ad5–PHPMA (decreased by 18%) and Ad5–AuPEG (decreased by 47%). These findings again confirm that KC-mediated capture is a crucial mechanism for the clearance Ad5–AuPEG from the blood stream. Notably, these data also indicate that passage of Ad5–AuPEG through the hepatic sinusoidal fenestrae (e.g. the other hepatic clearance mechanism) is more limited than for Ad5–PEG and Ad5–PHPMA. As these agents have the same hydrodynamic diameter (**Table 2.3**) this may indicate that the Au–PEG shield presents a less flexible shield than PEG or PHPMA.

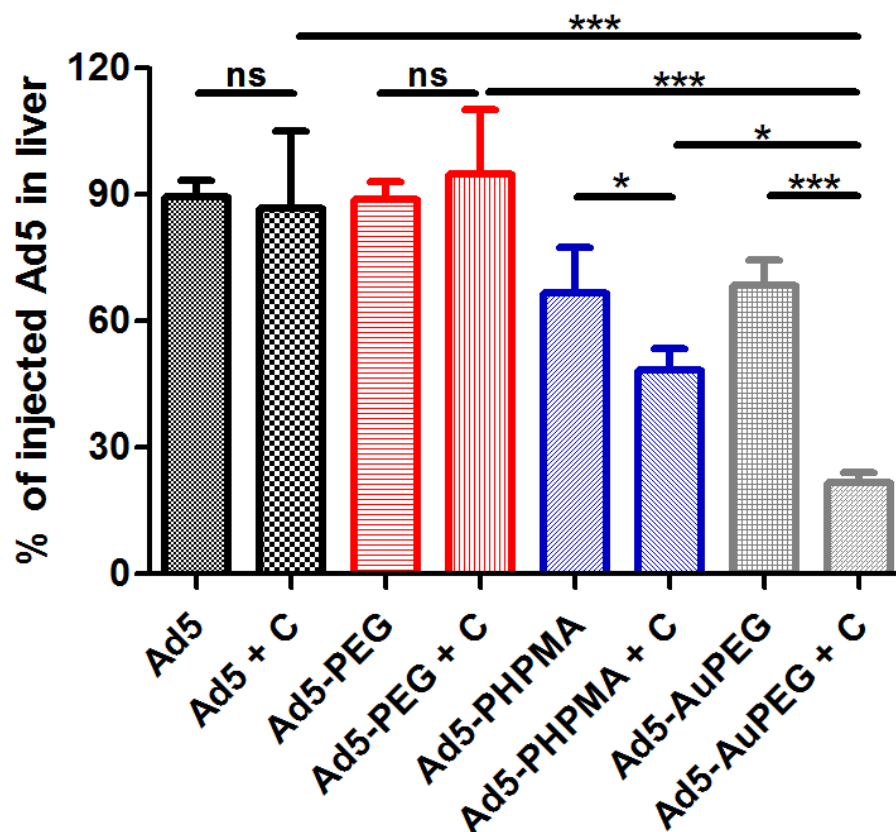


Figure 5.3: Total percentage of the injected Ad5 dose accumulated in livers. “C” represents administration of clodronate liposomes 24 hr before sample injection. Each group had four mice ($n = 4$), S.D. shown. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; ns = no significance; *, **, and *** represents p -value < 0.05 , 0.01 , and 0.001 , respectively.

Green *et al.* showed similar results with 97% of Ad5 and 81% of Ad5–PHPMA detected in liver for non-clodronate-treated mice after 30 min of circulation [118]. However,

to date, all literature published on Ad5 and Ad5-PHPMA only report hepatocyte infection data and not qPCR data for clodronate-treated mice. None of these papers measured the actual percentage of detectable Ad5 or Ad5-PHPMA by qPCR within the livers of clodronate-treated mice as these mice were used in a survival study [97, 119]. In addition, none of the published works on Ad5-PEG showed adenovirus quantification in liver, whether in control mice or clodronate-treated mice; the authors were only able to show a slight decrease in transgene expression of Ad5-PEG compared to Ad5 in non-clodronate-treated mice [113]. As a result, these transgene expression results could not be compared directly with the qPCR data obtained in this study. Consequently, for the first time, **Figure 5.3** not only summarises the actual percentage of Ad5 dosage across all Ad5 therapeutics but also shows a direct comparison of the liver accumulation among these Ad5 modifications, with and without clodronate treatment.

The total percentages of injected dose successfully delivered to tumour sites, 35 min after i.v. administration, are shown in **Figure 5.4**. The 0.13% and 0.04% of tumour accumulation achieved with Ad5 and Ad5 with clodronate, respectively, were comparable to the 0.07% of un-modified Ad5 accumulation in tumour at 6 hr post administration [113].

Despite having significantly longer plasma circulation time in the clodronate-treated mice than the control mice (**Figure 5.1**; $p < 0.05$), there was no significant difference in v.p. tumour accumulation between the clodronate-treated and un-treated mice for Ad5-PEG and Ad5-PHPMA groups ($p > 0.05$). However, the clodronate-treated mice did have a slightly higher (although not significantly so) percentage of tumour accumulation in each of these two groups: Ad5-PEG's tumour accumulation increased from 0.12% to 0.18% ($p > 0.05$), while Ad5-PHPMA's tumour accumulation increased from 0.50% to 0.62% ($p > 0.05$). This result was comparable to around 0.2% of Ad5-PEG accumulation (at 6 hr post-administration) detected in HEK 293 xenograft of non-clodronate-treated mice [113]. Green *et al.* also reported 0.7% of total Ad5-PHPMA detected in a clodronate-treated mouse HT29 xenograft

at 24 hr post-administration [97]. None of the published reports attempted to quantify Ad5-PEG or Ad5-PHPMA accumulated in mouse tumours at the 30-min time point. However, despite different tumour cell lines, mouse models, and time points of adenovirus quantification, the results in **Figure 5.4** and previously published studies showed similar levels of v.p. accumulation in tumours, with no study ever reporting a level in excess of 1% of the injected dose.

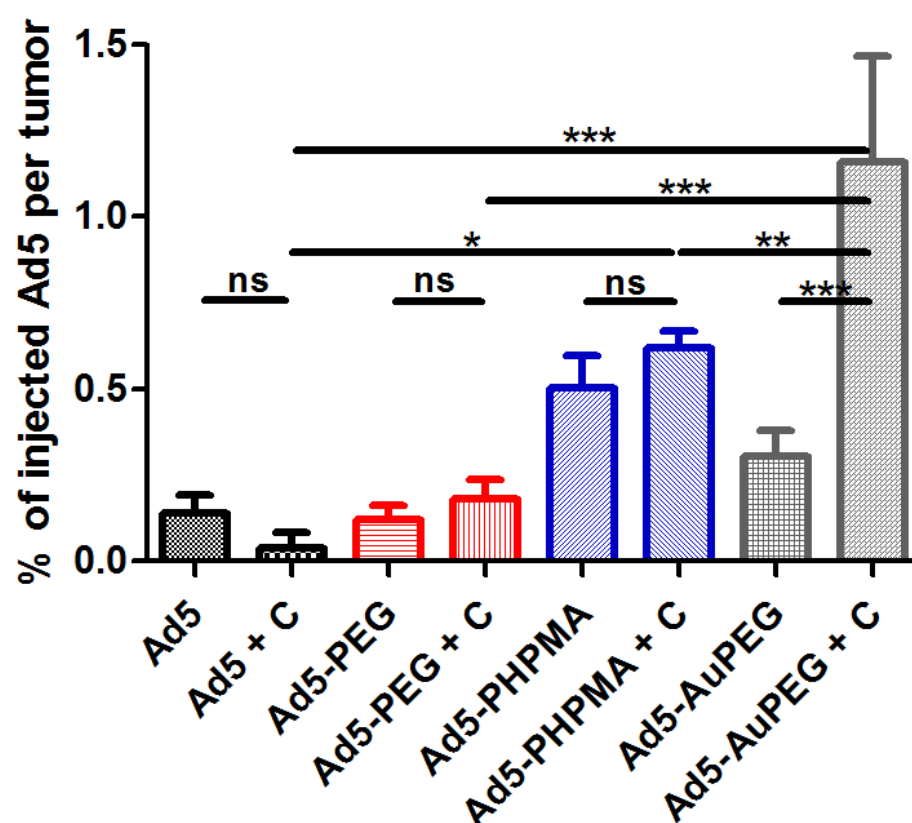


Figure 5.4: Total percentage of the injected dose accumulated per tumour. “C” represents administration of clodronate liposomes 24 hr before sample injection. Each group had four mice ($n = 4$), S.D. shown. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; ns = no significance; *, **, and *** represents p -value < 0.05 , 0.01 , and 0.001 , respectively.

In clodronate treated mice, 9-fold more Ad5-AuPEG + C (1.2% accumulation) than Ad5 + C were delivered to the tumour ($p < 0.001$). Furthermore, this delivery was significantly higher than the 0.18% or 0.62% achieved with Ad5-PEG + C or Ad5-PHPMA + C, respectively.

Integrating the areas under the curve (AUC) for each circulation profile (**Figure 5.1**) and plotting these data with their respective total Ad5 accumulated per gram of tumour (**Figure 5.4**) produced a strong correlation as shown in **Figure 5.5**. This line displayed a coefficient of determination, $R^2 = 0.6968$, indicating that passive tumour targeting of Ad5 is strongly dependent on its plasma AUC; this correlation was comparable to the $R^2 = 0.7149$ published previously [97]. Statically speaking, a good correlation R^2 value is > 0.6 ; a moderate correlation R^2 value is between 0.4 and 0.60; a weak correlation R^2 value is < 0.4 . Hence, it is likely that the improved tumour accumulation of Ad5–AuPEG via the EPR effect is a consequence of the extended circulation provided by its enhanced chemical coating and protection.

5.2.4 Implications for survival study and ultrasound therapy

Before investigating how ultrasound can best benefit the extravasation of v.p. into tumours, a good candidate needed to be pre-selected at this stage to reduce the number of mice used in line with the 3Rs: replacement, reduction, and refinement [272]. The data of plasma circulation, liver capture, and tumour accumulation all suggested that in the presence of clodronate treatment, Ad5–AuPEG was the superior candidate amongst Ad5, Ad5–PEG, and Ad5–PHPMA. Ad5–AuPEG was able to circulate for the longest duration (**Figure 5.1**), most efficiently avoid capture by the liver (**Figure 5.3**), and accumulate most effectively in tumours via the EPR effect (**Figure 5.4**). In addition, Ad5–AuPEG had the lowest binding to anti-Ad5 antibodies (**Figure 3.2**) and was extravasated the furthest in OptiCell® tumour-mimicking phantoms (**Figures 4.9** and **4.13**) using ultrasound parameters with known *in vivo* applicability [45]. Furthermore, the inclusion of a disulphide bond in the linkage between Ad5 and the AuPEG ensures that the activity of the Ad5 can be recovered in a tumour-triggered manner, and this re-activation mechanism was absent in both Ad5–PEG and Ad5–PHPMA.

As a result, five groups of mice were proposed for an *in vivo* survival study; PBS (negative control), Ad5 (positive virus control), Ad5 with inertial cavitation (positive ultrasound control), Ad5–AuPEG, or Ad5–AuPEG with inertial cavitation. All mice used in the survival study received clodronate liposome pre-treatment. All Ad5 therapeutics were co-injected with SonoVue® microbubbles to create cavitation activity induced by external ultrasound pressure.

5.3 *In vivo* testing of ultrasound-enhanced virotherapy

Having chosen Ad5–AuPEG as the best candidate to use for the investigation of ultrasound-enhanced virotherapy, the survival study required a few more modifications: to change the mouse model from BALB/c to CD1 mice, to use a human cancer xenograft instead of a syngeneic tumour model, and to replace the non-replicative Ad5 with a replication competent oncolytic Ad5 with specificity for human cancer cell lines. Since the chemistry modification used here was on the Ad5's surface, any oncolytic virus could potentially be a candidate for use with the AuPEG 'dandelion' structure.

It was decided that administrating Ad5-mir122 to treat HepG2 hepatocyte xenograft tumours would give the best therapy effects because of its very specific activity within these human liver carcinoma cells and reduced ability to replicate within murine liver [273, 274]. The 'mir122' denotes microRNA 122, which is present in high levels of normal liver cells and down-regulates excessive hepatocyte cellular division and activity [275]. However, many liver carcinoma cell lines have dramatically reduced expression of mir122 [276]. Cawood *et al.* genetically engineered Ad5-mir122 by introducing binding sites for mir-122 within the 3' un-translated region (UTR) of the early region 1A (E1A) transcription cassette within a wild type Ad5 genome. E1A is first Ad5 gene to be transcribed and responsible for amplifying and replicating the remaining of Ad5 genome. Given that Ad5 does not replicate in murine cells,

in healthy hepatocytes, the high levels of mir122 lead to effective binding to the mir122 sequence within the mir122-E1A of Ad5, reducing E1A levels and suppressing Ad5-mir122 replication. Conversely, in liver carcinoma, where mir122 expression is low, most Ad5-mir122 will not be down-regulated, resulting in successful replication of Ad5-mir122. In fact, Cawood *et al.* reported a 50-fold decrease of Ad5-mir122 replication compared to wild type Ad5 in normal mouse liver [273]. In addition, mir122 is conserved between vertebrate species [277], meaning the efficacy effects observed in mice have strong immediate application to clinical trials in humans.

The greatly improved efficacy achieved with Ad5-mir122 in the survival study reported by Cawood *et al.* used a combination of Ad5-mir122 and Ad5-mir122-luc [274]. In the study reported here, AuPEG was conjugated to a mixture of these two versions of Ad5-mir122. Ad5-mir122-luc was constructed by inserting luciferase gene immediately before the Ad5-mir122 binding site in the 3' UTR of E1A region. The function of Ad5-mir122-luc was to allow direct *in vivo* imaging of Ad5 expression in mice, thereby providing a means of tracking the development of the replication and spread of the Ad5 over time. It is notable however that the Ad5-mir122 is more potent than Ad5-mir122-luc [274]. Both Ad5-mir122 and Ad5-mir122-luc were gifts from Dr Ryan Cawood (Department of Oncology, University of Oxford).

5.3.1 Ultrasound setup, experimental methods, and mouse model

56 eight-week-old CD1 female mice were obtained from the Biomedical Services Unit, John Radcliffe Hospital (Oxford, UK). HepG2 cells (Sigma Aldrich, UK) were maintained using the cell culturing procedures described in section 3.2. For the convenience of naming, All 'Ad5' mentioned in sections 5.3.1 to 5.3.7 consists of 90% Ad5-mir122 and

10% Ad5-mir122-luc. At least 3×10^{11} copies of Ad5–AuPEG were synthesised and purified, as previously described in sections 2.3.

To establish HepG2 xenografts, each mouse was subcutaneously implanted with 100 μ L of DMEM containing 5×10^6 HepG2 cancer cells into the flank. After two weeks when the tumours reached size between 30 to 50 mm³, all 56 mice were pre-administrated with 150 μ L clodronate liposomes (Foundation Clodronate Liposomes, Netherlands). 24 hr later, all mice were randomly divided into one of four groups of four ($n = 4$) or one of five groups of eight ($n = 8$).

The experimental procedures described in this section were carried out in collaboration with Dr. Eleonora Mylonopoulou and Susan Graham. In particular, their development and optimisation of the equipment setup and ultrasound parameters employed in this *in vivo* study, as well as their expertise with therapeutic ultrasound, were instrumental to the success of this series of experiments. Most of the ultrasound experiments described in this section, the setup, hardware, and software are identical to those in section 4.1.2 and **Figure 4.3**, with modifications described below. Firstly, the alignment of mouse tumour with the HIFU transducer/PCD (confocally and coaxially aligned) was carried out by Z.One diagnostic scanner (Zonare, USA) with an L10-5 linear array probe (centre frequency 8 MHz, 6-cm maximum imaging depth, frame rate 14 Hz) that is in a 90° angle to the HIFU transducer and PCD (**Figure 5.6**). Secondly, each mouse was sedated with anaesthesia (2% isoflurane and 98% oxygen; flow rate at 2 L/min), submerged inside the water tank, setup with a tail vein cannulation, and i.v. dosed with PBS, Ad5, or Ad5–AuPEG with SonoVue® at time 0 min. Each mouse was intravenously (i.v.) dosed with 100 μ L PBS containing 25% SonoVue microbubbles (5×10^6 microbubbles) with no virus, 1×10^{10} copies of Ad5, or 1×10^{10} copies of Ad5–AuPEG per mouse at 0 min. Subsequently, the cannulation was used to deliver 25 μ L of SonoVue® at 2, 4, 6, 8, and 10 min while the mouse tumour was exposed for 12 min by the 0.5MHz HIFU transducer (PRFP 1250 kPa, 1.66% duty cycle, PRF 0.5 Hz) while the

PCD recorded all the cavitation signals from the tumour for the entire duration. Also at time 4 min and 8 min, the HIFU transducer and PCD were moved slightly (2 mm) to focus on two additional regions within the same tumour for each mouse, increasing the chance of targeting major tumour vasculature instead of possibly necrotic regions. During this treatment period, half of the mice received ultrasound treatment as described above, and half the mice did not (the arrows in **Figure 5.8** represent the dosing time line). The 37°C temperature of the water in the tank (**Figure 5.6**) was maintained by using an immersion heater (GD100; Grant Instruments, UK). **Figure 5.7** uses flow charts to summarise the whole experiment of ultrasound-enhanced virotherapy.

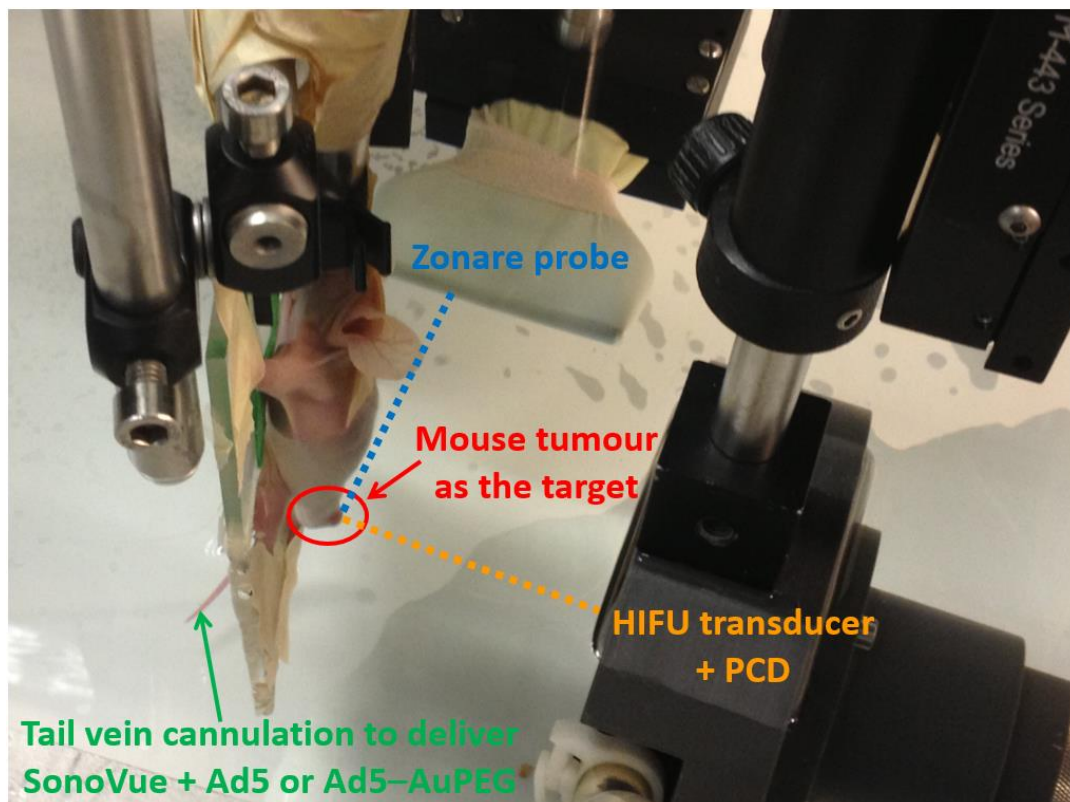


Figure 5.6: Experimental setup to align HIFU transducer and PCD with the mouse tumour by Zonare probe. Inside a water tank, the Zonare probe formed a 90° angle to the HIFU transducer and PCD, which were confocally and coaxially aligned. While the tumour was under ultrasound exposure, SonoVue® microbubbles with Ad5 or Ad5–AuPEG were delivered using the cannulation tube set up in the tail vein of each mouse.

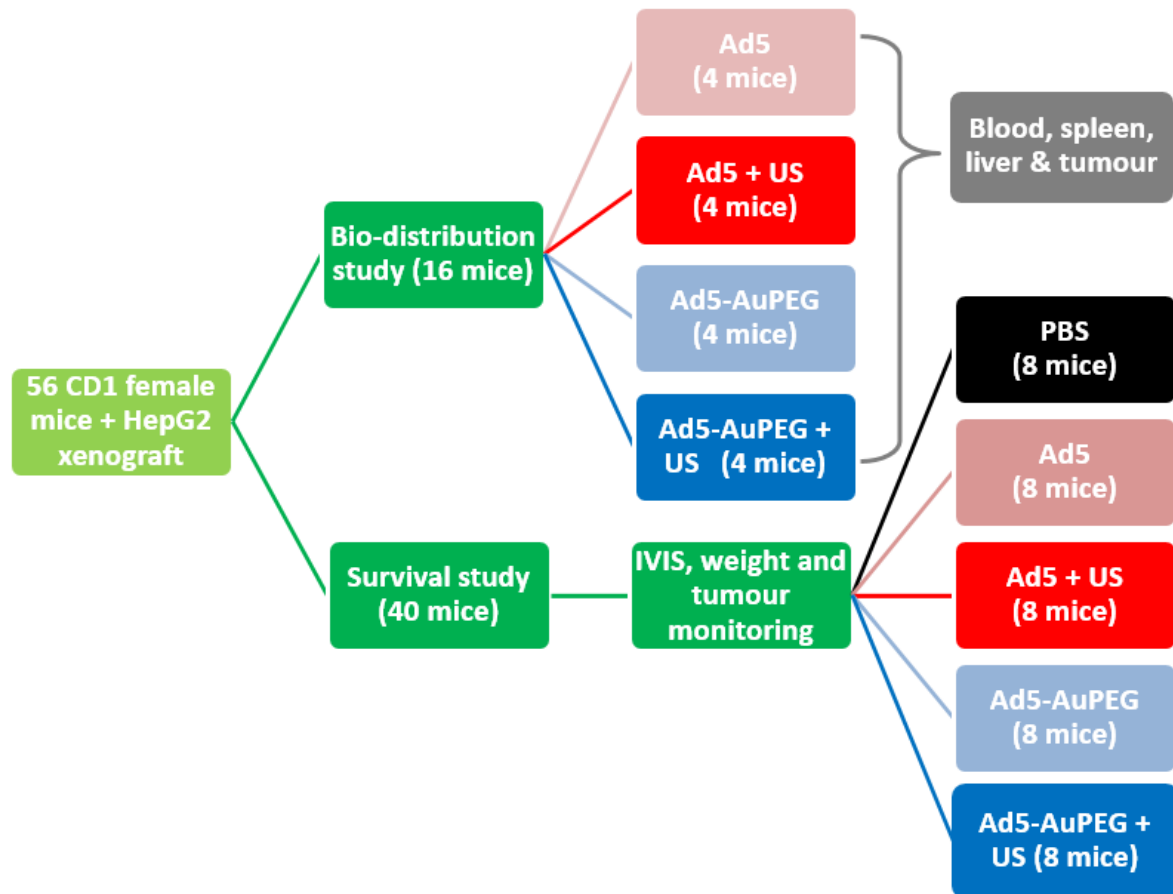


Figure 5.7: Experimental summary of the ultrasound-enhanced virotherapy. Total 56 CD1 nu/nu female mice were divided into four groups of four ($n = 4$) and five groups of eight ($n = 8$) for bio-distribution study and survival study, respectively. qPCR was used to quantify Ad5 accumulation in blood, spleen, liver, and tumours 30 min after the bio-distribution experiment, while the 40-mice survival study lasted for around three weeks. ‘US’ denotes ultrasound treatment, as describe in section 5.3.1. This allowed the pharmacokinetics and biodistribution of Ad5–AuPEG to be characterised in a second model and the effect of ultrasound on these aspects to be tested in addition to the effects on survival. The survival study was only performed once.

Besides optimising the PRF value to 0.5 Hz to allow enough time for SonoVue® to replenish the tumour vessels, Bazan-Peregrino *et al.* had previously used the same ultrasound parameters to instigate inertial cavitation in mouse xenograft tumours [45, 261]. As a result, all ultrasound parameters were chosen based on previous work done by Bazan-Peregrino *et al.* with one exception: Bazan-Peregrino *et al.* only re-injected SonoVue® once at 2 min. The rationale for this was that beyond this early time-point Ad5 would have cleared to the liver and would not be available in the bloodstream for ultrasound assisted extravasation into the tumour [45]. Here SonoVue® was replenished at 2, 4, 6, 8, and 10 min because previous data in section 5.2 had demonstrated Ad5–AuPEG could circulate much longer than un-modified

Ad5, and so continued exposure still had the potential to provide benefit. For the mice without ultrasound treatment, the same SonoVue® microbubbles and Ad5 or Ad5–AuPEG were delivered in the same manner, except tumours were not exposed to ultrasound, however mice were still submerged inside the 37°C water tank.

For the 16 mice assigned for the bio-distribution study, 20-µL blood samples were taken from the tail vein at 30 min after Ad5 administration, diluted to 200 µL in PBS, and stored at -20°C immediately. Tumour, liver, and spleen samples were extracted following cull at 30 min and homogenised using an Ultra-Turrax homogenizer (model T18; IKA Laboratory Equipment, Germany) in PBS at the concentration of 125 mg/mL. DNA was extracted from blood and homogenised tissue samples using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, UK), as previously described in section 3.3.1. qPCR was used to detect the presence of Ad5 DNA in all extracted DNA samples, as previously described in section 3.3.1.

For the remaining 40 mice assigned for the survival study, tumours were sized and mice weighed each day. Tumour volume was measured using an electronic digital caliper (Maplin, UK) and calculated as an ellipsoid by the formula $\text{volume} = \frac{1}{2}(\text{length} \times \text{width} \times \text{height})$ [278]. The mouse was culled by following two criteria: firstly if the tumour volume exceeded 1000 mm³; secondly if the weight loss was more than 15% of the original weight on the day of the tumour cell implantation. To track luciferase expression within livers and tumours, at days 1, 2, 7, and 14, mice were intraperitoneally injected with 2 x 50 µL of 15.8 mg/mL D-luciferin (Sigma Aldrich, UK) in PBS. 4 min after the luciferin injection, murine liver and tumour were imaged using an IVIS 100 imaging system (Xenogen, USA) to detect *in vivo* virus infection.

The statistical analyses of bio-distribution and tumour growth data in this chapter were completed using a one-way analysis of variances (ANOVA) followed by a Newman-Keuls

test for pairwise comparison of sub-groups. For the statistical analysis of the survival study, a Mantel–Cox log-rank test was used to determine the significant p values and hazard ratios. This statistic test was selected because it is widely used in most clinical trials for survival study to establish the efficacy of a new treatment compared to a control treatment. All data are reported such that *, **, and *** represent p-value < 0.05, 0.01, and 0.001, respectively.

5.3.2 Results: cavitation data captured by PCD

The 12-min cavitation activity recorded by the PCD for each treated tumour (**Figure 5.8**) showed several characteristics of the power spectra which include a sudden rise in broadband emissions upon injection and re-injection of SonoVue® microbubbles, and persistence of inertial cavitation with time for 70 sec on average, which was comparable to the 80-sec half-life of SonoVue® reported previously [45].

While **Figure 5.8** only shows the cavitation data of four mice, this is a typical representation of all the cavitation data captured for all 24 mice that received the same ultrasound treatment. Importantly, no difference ($p > 0.05$) in cavitation activity was detected in mice treated with Ad5 versus mice treated with Ad5–AuPEG. Furthermore, harmonic emissions (corresponding to stable cavitation) were not detected in any of the ultrasound-treated mice, suggesting all SonoVue® microbubbles administrated into the blood stream were rapidly destroyed and yielded inertial cavitation.

No quantitative relationship (linear regression line $R^2 = 0.1343$) was found between the inertial cavitation dose detected (measured by total broadband energy) and the level of v.p. delivery to tumour (measured by qPCR), an observation also made by Bazan-Peregrino *et al.* [45]. The variation in tumour vasculature development, percentage of necrotic region, and extent of the tumour area targeted by ultrasound may explain this lack of relationship. As the HIFU transducer used in this study may create non-uniform exposure over the whole tumour volume, enhanced viral delivery and infection may take place in tumour regions outside the

direct focal volume. Since the beam profile of the HIFU focus has a full-width half-maximum of 30 mm axially and 3 mm transversally, the majority of the 30- to 50-mm³ tumour volumes were treated during the ultrasound exposure.

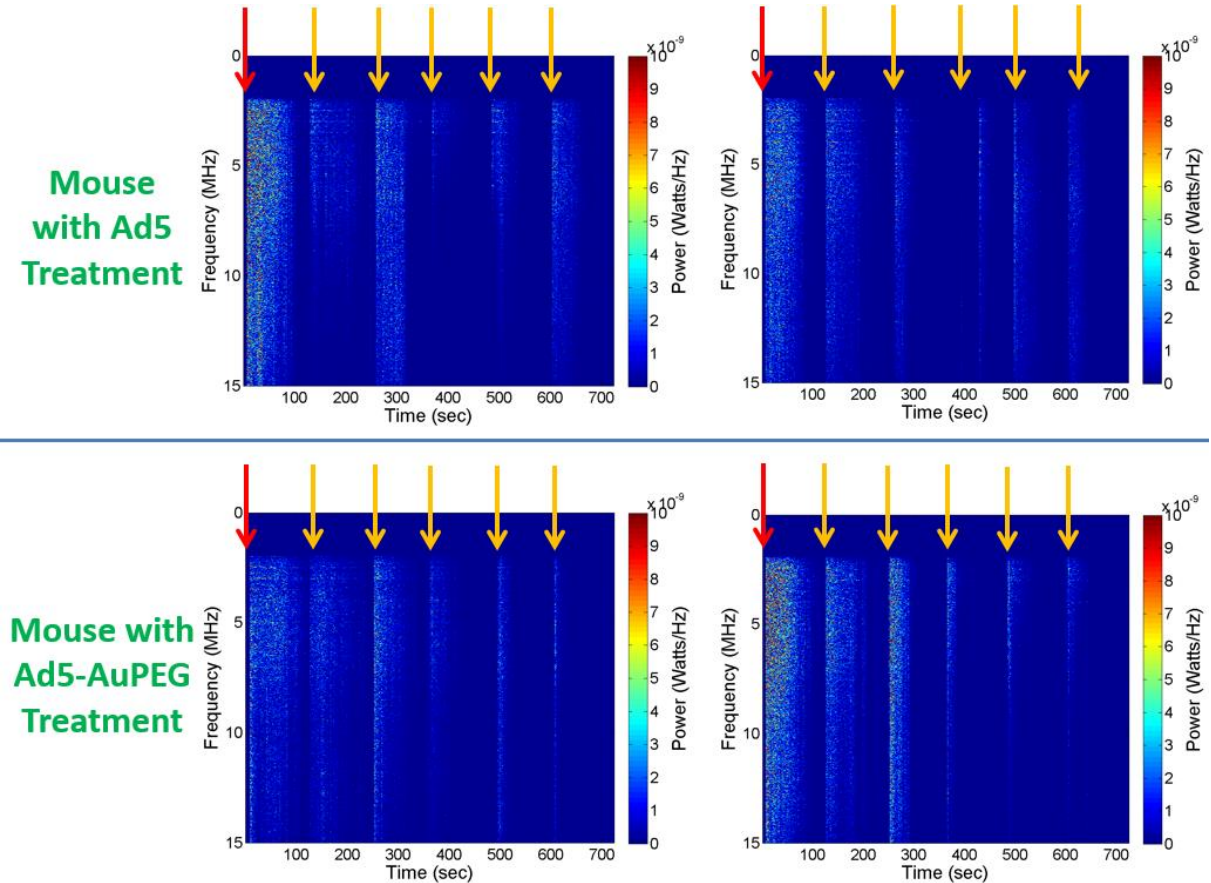


Figure 5.8: Spectrograms of passive cavitation data acquired from four HepG2 xenograft mice. There was sustained broadband emissions throughout the 12-min exposure at 508 kHz, 1.25 MPa, 1.66% duty cycle, 0.5 PRF ($n = 24$). The red arrows represented time = 0 min when both SonoVue® microbubbles and Ad5 or Ad5–AuPEG were administered, and the orange arrows indicated the subsequent injection of SonoVue® only at 2-min intervals.

5.3.3 Results: bio-distribution of Ad5 and Ad5–AuPEG with ultrasound

The bio-distribution data obtained from this survival study experiment added three major components compared to the bio-distribution data shown in section 5.2.3. Firstly, virus accumulation was detected in human carcinoma HepG2 xenografts; secondly, ultrasound-enhanced effects were added; thirdly, spleen was also extracted in this study to compare virus captured by spleen versus liver. **Figure 5.9** summarises the findings in terms of percentage of injected dose per mouse (10^{10} copies of v.p.).

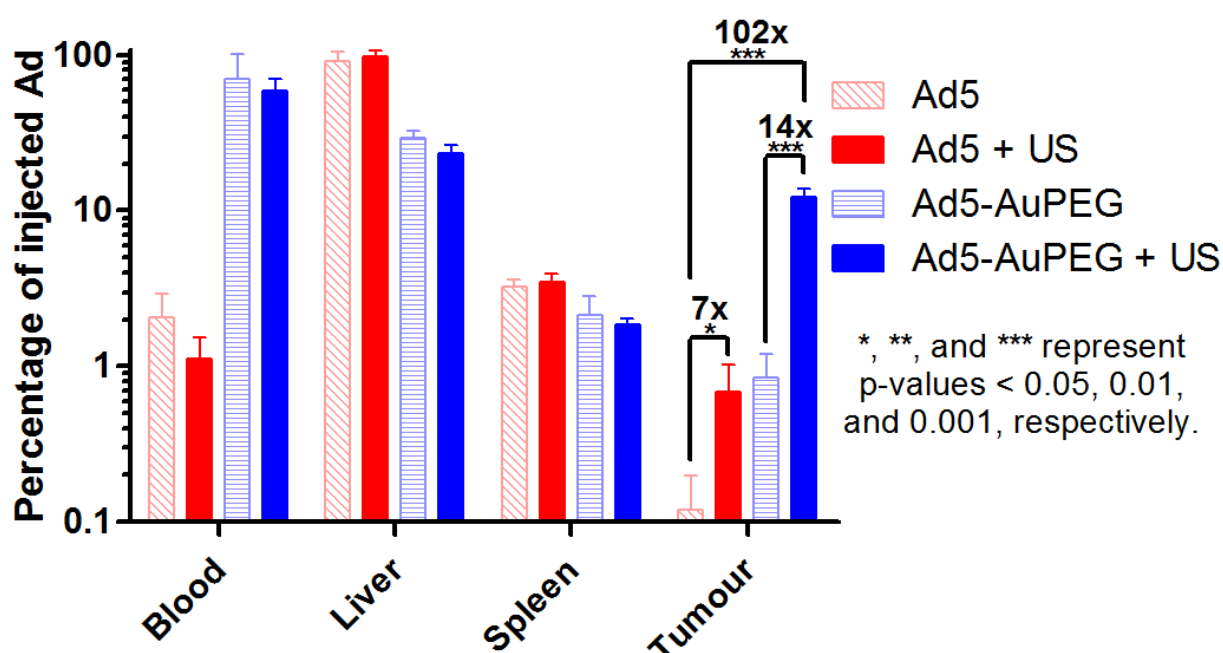


Figure 5.9: Total percentage of the injected virus dose accumulated in blood, liver, spleen, and tumour 30 min after treatment. All mice were treated with clodronate liposomes 24 hr before the virus $\pm$ ultrasound dosing. Each group had four mice ($n = 4$), S.D. shown. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; *, **, and *** represents p -value < 0.05 , 0.01 , and 0.001 , respectively. US = ultrasound treatment.

The 30-min bio-distribution for blood, liver, and tumour in the absence of ultrasound-treatment were at similar levels as the bio-distribution data shown in **Figures 5.1 to 5.4** and previously published studies as discussed in section 5.2. When the ultrasound treatment was given, the total plasma circulating Ad5 dropped from 2.1% to 1.1% (although this decrease was found to be non-significant; $p > 0.05$) and total circulating Ad5–AuPEG dropped from 69% to 58% (also $p > 0.05$). Interestingly, this loss in plasma circulation Ad5 and Ad5–AuPEG was reflected by an increase of their accumulation in the tumour. In the presence of ultrasound treatment, Ad5 increased its tumour accumulation 7-fold ($p < 0.05$) from 0.12% to 0.69% while Ad5–AuPEG boosted its tumour accumulation 14-fold ($p < 0.001$) from 0.84% to 12%. Bazan-Peregrino *et al.* failed to show significant increases in tumour accumulation of Ad5 upon application of ultrasound. This difference may be explained by the fact that in that the dose was fractionated into two injections of 5×10^9 v.p. in the studies, which may have provided a still lower circulating dose within the tumour for the ultrasound to impact upon. [45]. Using similar dose fractionation, Carlisle *et al.* also demonstrated no increase in Ad5

tumour deposition using ultrasound but did demonstrate Ad5–PHPMA tumour accumulation could be improved by 2 fold, which translated into a 30-fold increase in transgene expression at 72 hr [279].

Whether with ultrasound treatment or not, the 30-min liver accumulation of Ad5 and Ad5–AuPEG remained the same ($p > 0.05$) at 90% and 25%, respectively; in addition, Ad5–AuPEG liver accumulation was significantly lower than that of Ad5 ($p < 0.001$), as previously observed in section 5.2. Ultrasound treatment did not affect Ad5 or Ad5–AuPEG accumulation in spleen ($p > 0.05$) which remained at 3% and 2%, respectively. The slightly lower percentage of spleen accumulation from Ad5–AuPEG arose directly from the longer plasma circulation time. The level of Ad5 detected in spleen without the ultrasound treatment was comparable to the 2.5% level reported by Green *et al.* [118]. In summary, although spleen did serve as another tissue to trap Ad5 and Ad5–AuPEG, the level of virus which accumulated was 30-fold lower than that in liver.

5.3.4 Results: IVIS imaging on liver and tumour

After dosing, liver and tumour IVIS images were taken on days 1, 2, 7, and 14. **Figure 5.10** (in log scale) shows the average light intensity (photons/sec/cm²) from the liver of all the mice. These data show that there was a gradual decrease in the level of liver infection over the period of the survival study. The infection levels of Ad5 and Ad5–AuPEG (whether with ultrasound treatment or not) were similar to each other and always expressed at a significantly higher level ($p < 0.001$) than liver signals obtained from control PBS mice. The light intensity levels shown for these Ad5-mir122-luc were comparable to previous studies and always around two orders of magnitude lower than those reported for a non-mir containing replication competent Ad5-luc [273]. This indicates that although the majority of oncolytic Ad5 and Ad5–AuPEG were captured by the liver at day 0, these Ad5-mir122-luc v.p. did not

replicate effectively in the liver due to the high presence of mir122 to interfere the transcription of the viral genome.

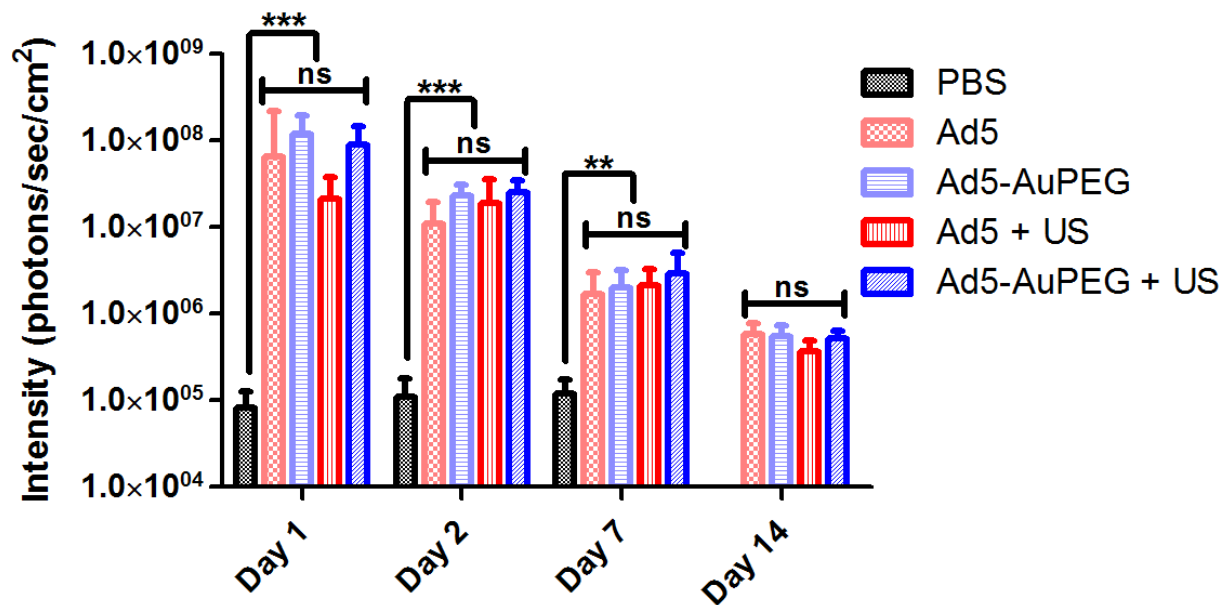


Figure 5.10: Light intensity measured for Ad5 and Ad5–AuPEG liver infection over two-week period after treatment. Each group had eight mice ($n = 8$) for days 1 and 2 to start out with, S.D. shown. Days 7 and 14 show data acquired from the remaining survival mice. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; ns = no significance; *, **, and *** represents p -value < 0.05 , 0.01 , and 0.001 , respectively. US = ultrasound treatment.

The level of luciferase expression within tumours, as measured by IVIS imaging, is shown in **Figure 5.11** in linear scale. For day 2, the transgene expression for Ad5, Ad5–AuPEG, Ad5 + US, and Ad5–AuPEG + US compared with PBS-treated tumours increased by factors of 1.9 ($p < 0.05$), 1.6 ($p < 0.05$), 3.7 ($p < 0.001$), and 3.1 ($p < 0.001$), respectively. Notably at day 2, in both the presence and absence of ultrasound treatment, the level of transgene expression detected between Ad5 and Ad5–AuPEG showed no significant difference ($p > 0.05$), although ultrasound treatment was shown to improve the transgene expression of both Ad5 and Ad5–AuPEG by a factor 2 ($p < 0.01$). These data suggested ultrasound treatment not only improved Ad5 and Ad5–AuPEG’s tumour accumulation (**Figure 5.9**) but also enhanced their transgene expression.

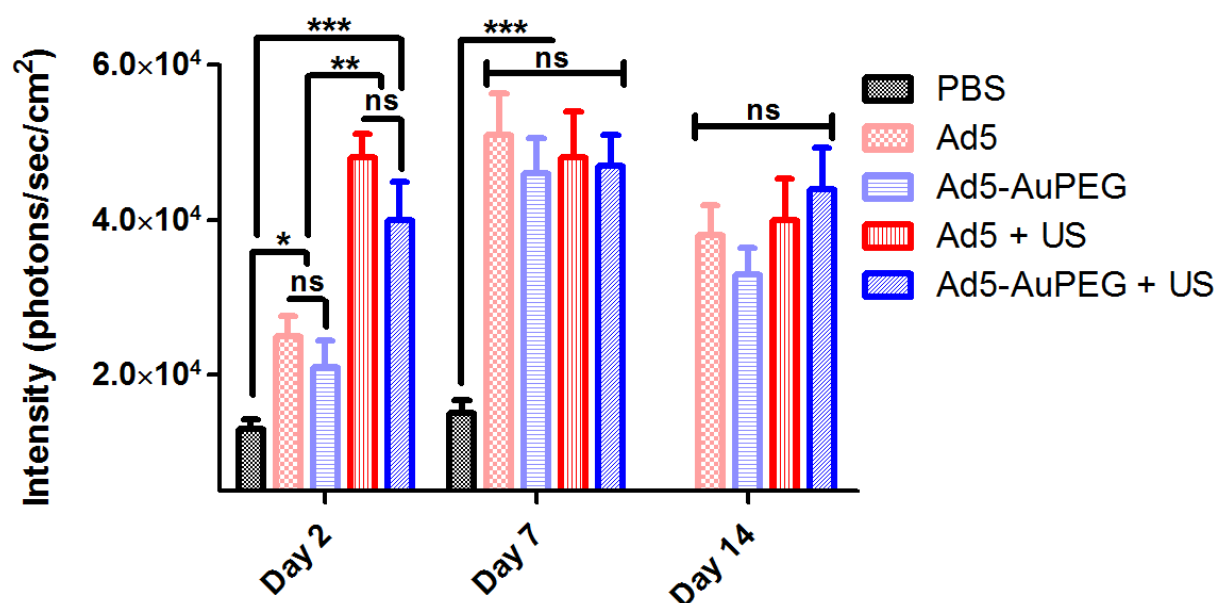


Figure 5.11: Light intensity measured for Ad5 and Ad5–AuPEG tumour infection over two-week period after treatment. Each group had eight mice ($n = 8$) for day 2 to start out with, S.D. shown. Days 7 and 14 were data acquired from the remaining survival mice. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; ns = no significance; *, **, and *** represents p -value < 0.05 , 0.01 , and 0.001 , respectively. US = ultrasound treatment.

Interestingly, given that these viruses were oncolytic, at days 7 and 14, there was no significant difference in tumour luciferase expression ($p > 0.05$) detected between ultrasound-treated tumours and non-treated tumours. Indeed, the level of transgene expressions stayed virtually at the same magnitude of 10^4 photons/sec/cm² as day 2. While Carlisle *et al.* were able to demonstrate a 30-fold increase in tumour transgene expression of adenovirus at 72 hr using ultrasound [45, 279], 100% of the virus used in these studies carried luciferase gene. The lack of apparent difference in tumour transgene expression in the studies reported here could be a result of the fact that only 10% of the total injected virus dose carried the luciferase gene. Furthermore, in contrast to the study of Cawood *et al.*, a smaller dose of virus was administered in this study (1×10^{10} v.p.). Cawood *et al.* had observed tumour transgene expression at a dose of 8.6×10^{10} v.p. without ultrasound, however if the same dose had been used here the influence of ultrasound could not have been delineated for the intrinsic ability of the virus to infect and kill tumour cells. Indeed, the injection of one single low dose of Ad5, such as 1×10^{10} v.p. in this study, is unusual; in most Ad5 virotherapy studies multiple

injections were made [97, 274]. It is also notable that, the HepG2 xenograft implanted in this study grew at a much more aggressive rate (see section 5.3.5) than previously reported for this or other human xenografts. Such increased growth rate may prevent the replication, and spread of the virus outstripping the growth of the cells. It is also possible that ‘competition’ between the 90% Ad5-mir122, and the less efficient 10% Ad5-mir122-luc lead to loss of the luciferase expressing virus. Since Ad5-mir122-luc was less potent than Ad5-mir122 [274], it was safe to assume Ad5-mir122 can dominate competition for replication resources.

5.3.5 Results: tumour development and weight loss

There are many factors that can influence the growth rate of xenografts on CD1 nude mice, including batch-to-batch variation in cell line growth rate and virus susceptibility, genetic drift in strains of laboratory mice, and the age of mice at the time of cancer cell implantation. Previous studies have shown it took anywhere from 3 weeks [280] or 4 weeks [281] to 8 weeks [282] for HepG2 tumour growth, in the absence of any treatment, to reach 1000 mm³ after the initial inoculation of these cancer cells. For this study, the ultrasound and virotherapy treatments were applied 15 days after the initial implantation of HepG2 cells.

Figure 5.12 shows the tumour development over time, with the horizontal axis representing the number of days after treatment. As described earlier in section 5.3.1, the initial tumour volumes at the day of the treatment were between 30 to 50 mm³. Firstly, it took 4 weeks (including the days before treatment) for the HepG2 tumour in our control PBS mice to reach over 1000 mm³, and this result was comparable to previous published data for more aggressive HepG2 development [280, 281]. One week after the treatments, significantly reduction ($p < 0.05$) in tumour growth rate were observed for both Ad5 + US and Ad5–AuPEG + US treatment groups compared to the control PBS group. At day 10 and beyond, all treatment groups (except Ad5–AuPEG) were able to suppress the tumour growth rate significantly ($p < 0.05$, compared to PBS).

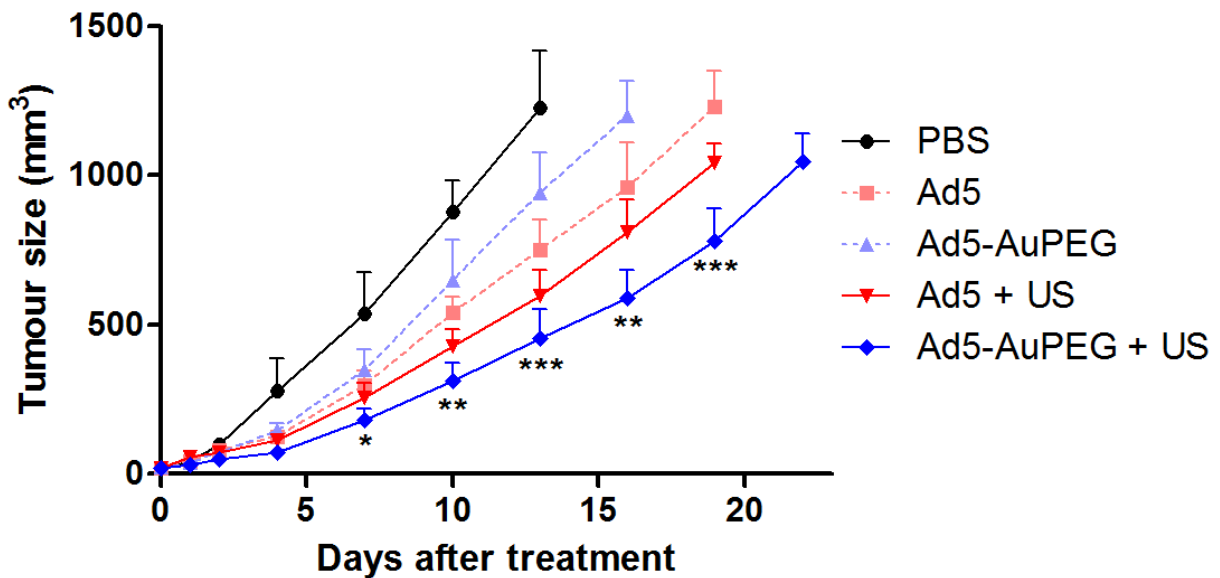


Figure 5.12: The progression of HepG2 tumour growth after ultrasound $\pm$ virotherapy treatment. Ad5 = 90% of Ad5-mir122 + 10% of Ad5-mir122-luc; each group had eight mice ($n = 8$), S.D. shown. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; ns = no significance; *, **, and *** represents p -value < 0.05 , 0.01 , and 0.001 , respectively. The star(s) for statistical significance represent the comparisons between Ad5–AuPEG with the largest tumour group in each given day.

Tumour growth curve comparisons	Ratio of AUC of tumour growth	Sig different? $P < 0.05$?
PBS vs. Ad5	1.69	Yes
PBS vs. Ad5–AuPEG	1.41	Yes
PBS vs. Ad5 + US	2.05	Yes
PBS vs. Ad5–AuPEG + US	2.84	Yes
Ad5 vs. Ad5–AuPEG	0.82	No
Ad5 vs. Ad5 + US	1.20	No
Ad5–AuPEG vs. Ad5–AuPEG + US	2.03	Yes
Ad5 vs. Ad5–AuPEG + US	1.65	Yes
Ad5 + US vs. Ad5–AuPEG + US	1.37	Yes

Table 5.13: Ratios of area under the curves (AUC) from all tumour growth lines. AUC for each tumour progression lines in Figure 5.12 were first integrated and then compared with each in terms of ratios showed in this table. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; if $p < 0.05$, then the two groups were statistically significant.

As previously discussed, **Figure 5.9** demonstrated that the 30-min tumour accumulation percentages for Ad5, Ad5–AuPEG, Ad5 + US, and Ad5–AuPEG + US were 0.12%, 0.69%, 0.84%, and 12%, respectively. Despite Ad5–AuPEG having 7-fold higher

initial tumour accumulation than Ad5, the former did not suppress the tumour growth as well as the latter. From our *in vitro* experiments (**Figure 3.3**), the reactivation of Ad5–AuPEG in an idealised tumour-like reducing environment gave a maximum of 42% infection recovery. In this *in vivo* study, it was unlikely that all extravasated Ad5–AuPEG was located in high reducing environment within the tumour, especially as without ultrasound its accumulation would be driven by the EPR effect and lead to accumulation in the perivascular space only. A failure to be reactivated may therefore explain how in the absence of ultrasound more Ad5–AuPEG gave lower efficacy than Ad5. Notably the tumour growth lines of Ad5 + US and Ad5–AuPEG + US were also close to each other, despite the fact that 17-fold more Ad5–AuPEG + US accumulated in the tumour than Ad5 + US. This indicates that even if Ad5–AuPEG could be driven deeper into the tumour, AuPEG release and virus re-activation was still not optimal. However, the tumour growth rate of Ad5–AuPEG + US was still significantly slower than that of Ad5 + US ($p < 0.05$).

A previous study by Cawood *et al.* demonstrated that the same treatment regime of 90% Ad5-mir122 and 10% Ad5-mir122-luc not only retarded the tumour growth but actually suppressed the tumour volume to make them shrink over time [274]. However, the authors injected a total of 8.6×10^{10} copies of Ad5 (versus 1×10^{10} copies of Ad5 in this study), administered the Ad5 three times at days 0, 3, and 6 (versus one single injection at day 0 in this study), started treating the mice when the tumour volumes were only 10 to 20 mm³ (versus initial tumour volume of 30 to 50 mm³ at the day of the treatment), and had a much slower growing control HepG2 tumour that reached 500 mm³ in four weeks (versus the control HepG2 tumour that reached 500 mm³ in one week in this study) [274]. These comparisons explain why retardation of growth, rather than regression of tumour size, was seen in the studies reported here.

By integrating the area under the curve (AUC) for all five tumour average growth lines in **Figure 5.12**, these AUCs could provide a level of longitudinal analysis and be compared to

assess the overall tumour growth inhibition efficacy of the four treatments compared to the PBS control. **Table 5.13** shows the ratios of larger AUC to smaller AUC for all possible pair comparisons.

In this study, the ‘effective tumour reduction ratio’ was defined as the degree to which growth could be hindered for a given treatment compared to the non-treated control group. While a ratio > 1 means reduced tumour growth, a ratio < 1 indicates increasing tumour growth. The least useful treatment was Ad5–AuPEG alone with an effective tumour reduction ratio of 1.41 (although it was still significant; $p < 0.05$) compared to PBS, and the best treatment was the combination of Ad5–AuPEG with ultrasound with an effective tumour reduction ratio of 2.84 ($p < 0.001$). Notably with ultrasound treatment, the effective tumour reduction ratio improved by 20% ($p > 0.05$) for Ad5 and 103% ($p < 0.001$) for Ad5–AuPEG, again confirming our previous *in vitro* and bio-distribution conclusion that Ad5–AuPEG was much more responsive to therapeutic ultrasound due to its increased density. Lastly, coating the surface of Ad5 with AuPEG (making Ad5–AuPEG) actually increased the tumour growth with a reduction ratio of 0.82, the only ratio that is < 1 in this study. This suggested that although higher dose of Ad5–AuPEG passively accumulated in tumour than un-modified Ad5 (**Figure 5.9**), majority of the Ad5–AuPEG were unable to be reduced at the tumour environment, resulting lower viral infection in the tumour and making Ad5–AuPEG therapy actually worse than Ad5 alone.

The body weights of all mice were monitored throughout the entire survival study. Before tumour cells implantation, all the mice were mature, eight-week old, and had maintained steady weight for at least one week. The body weights on the day of treatment (day 0, which was 15 days after the initial tumour inoculation) were defined as 100% (ranged from 25 g to 28 g), and subsequent body weights recorded were normalised based on the day 0 weight for each mouse. **Figure 5.14** shows the change in body weight for the five treatment groups over time. Considering the variation of body weight within each group, there was

neither a significant gain nor loss ($p > 0.05$) of body weight for the first 2.5 weeks after the treatment. However, significantly lower body weights ($p < 0.05$) were observed for the surviving mice on day 19 (an average of 8% loss of initial weight) and onward. During the entire survival study, only four mice were culled based on the ‘more than 15% of body weight loss’ rule: one mouse from each of the four treatment groups and none from the control PBS group. The vast majority of the mice were culled when the tumour size exceeded 1000 mm³.

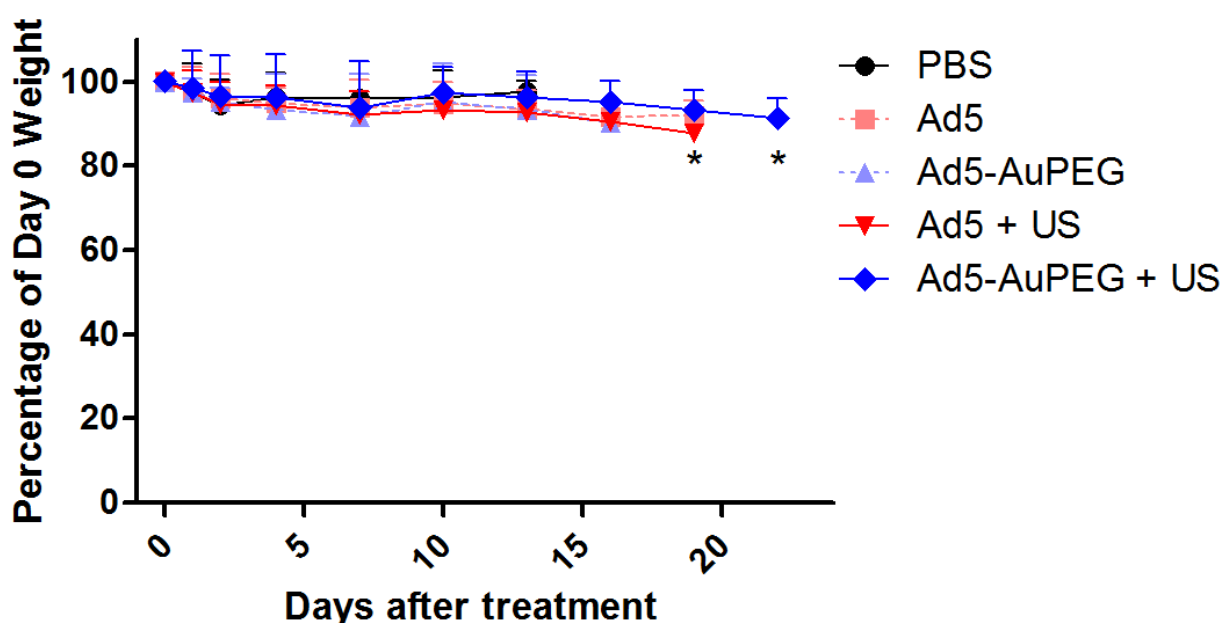


Figure 5.14: Mouse body weight changed after the treatment. All data points were normalised to day 0 body weights, which were defined as 100%. Each group had eight mice ($n = 8$). * means $p < 0.05$, indicating the body weights measured were significantly lower than the day 0 body weight.

5.3.6 Results: survival study

Mouse survival data were input into the GraphPad Prism software (version 5.0.2; GraphPad Software, USA), and five Kaplan–Meier survival curves were generated by to compute the percentage of survival for each group at any given time point (day) following the treatments (**Figure 5.15**). Mantel–Cox log-rank test for each pair of survival curves was also performed to obtain p-values and hazard ratios for all comparisons (**Table 5.16**).

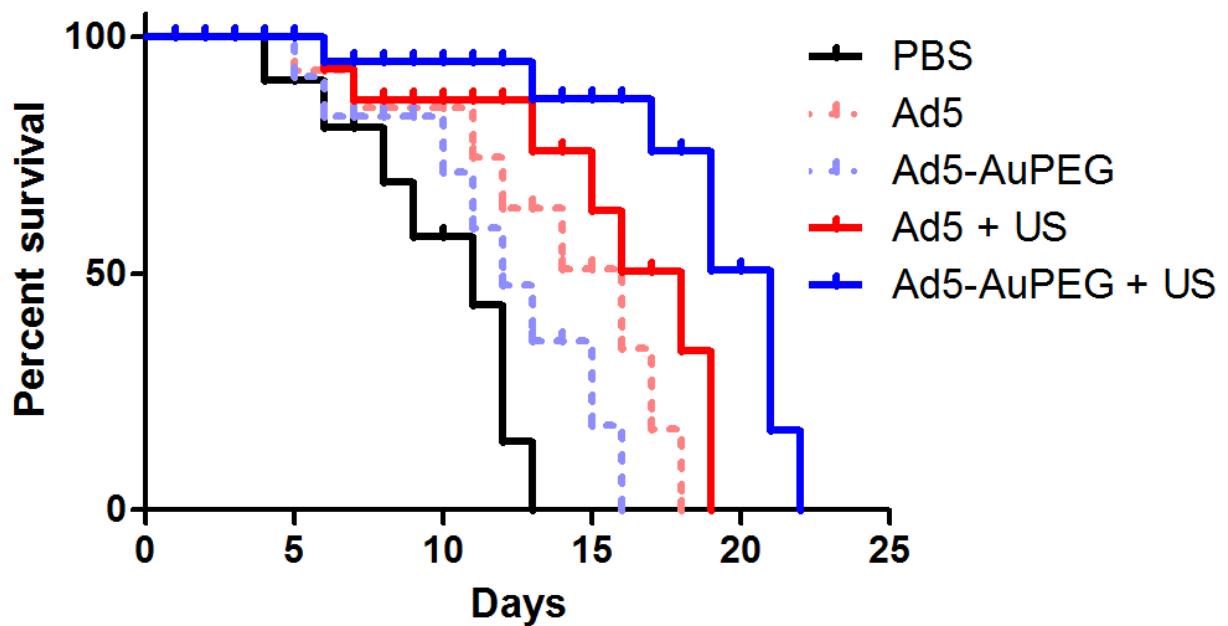


Figure 5.15: Kaplan-Meier survival curves of mice with HepG2 carcinoma treated with virotherapy ± therapeutic ultrasound. Five experimental groups (1 control and 4 treatments) were studied for the efficacy of Ad5 or Ad5–AuPEG, with or without ultrasound treatment. Each group had eight mice ($n = 8$). A mouse was culled when its tumour size $> 1000 \text{ mm}^3$ or weight loss more than 15% of the original weight measured on the day of the treatment.

Log-rank Test for Survival Curves	P value	Sig different?	Hazard ratio
PBS vs. Ad5	0.0134	Yes	4.84
PBS vs. Ad5–AuPEG	0.1277	No	2.46
PBS vs. Ad5 + US	0.0009	Yes	9.97
PBS vs. Ad5–AuPEG + US	< 0.0001	Yes	32.41
Ad vs. Ad5–AuPEG	0.2055	No	2.03
Ad vs. Ad5 + US	0.1645	No	2.21
Ad5–AuPEG vs. Ad5–AuPEG + US	0.0023	Yes	15.92
Ad5 vs. Ad5–AuPEG + US	0.0017	Yes	8.26
Ad5 + US vs. Ad5–AuPEG + US	0.0263	Yes	4.07

Table 5.16: Statistical results of Mantel–Cox log-rank test for all survival curves. If the p value is < 0.05 , the two survival curves are significantly different from each other. The hazard ratio for each comparison was also calculated.

The survival curve analysis indicated that the half-life (50% survival rate) of mice treated with PBS, Ad5, Ad5–AuPEG, Ad5 + US, and Ad5–AuPEG + US were 11 days, 16 days, 12 days, 18 days, and 21 days, respectively. While the treatment with Ad5 or Ad5–AuPEG increased the half-life of the control mice by 45% and 9%, respectively, the treatment

with Ad5 + US or Ad5–AuPEG + US enhanced the half-life by 64% and 91%, respectively. The order of these five survival curves (from the least to most surviving curve; **Figure 5.15**) matched the order of the tumour progression curves (from fastest to slowest growing curve; **Figure 5.12**).

The results of the log-rank test showed that with the exception of Ad5–AuPEG ($p > 0.05$), all treatment groups (Ad5, Ad5 + US, and Ad5–AuPEG + US) provided significant enhancement of survival compared to the control PBS group ($p < 0.05$). Although the ultrasound treatment did not improve the survival probability of Ad5 ($p > 0.05$), it did for Ad5–AuPEG ($p < 0.01$) by increasing the half-life of Ad5–AuPEG-treated mice by 75%.

Hazard ratio is a measurement of instantaneous risk at any given time period and serves as a better measurement to compare two surviving groups treated with different therapeutics [283]. A hazard ratio therefore is another way to indicate the fold-increase in survival probability. The hazard ratios of Ad5, Ad5–AuPEG, Ad5 + US, and Ad5–AuPEG + US to PBS mice were 4.84, 2.46, 9.97, and 32.4, respectively; this suggests that, at any given time point, mice treated with Ad5 + US or Ad5–AuPEG + US had 10 and 32 times, respectively, more likelihood to survive than mice treated with PBS. Comparing the results of Ad5 and Ad5–AuPEG + US gave a hazard ratio of 8.26 and a significant p-value of 0.0017, suggesting the value-added of AuPEG and ultrasound enhances survival probability by a factor of 8.26 compared to the virus alone. This can be, compared to a hazard ratio of 2.21 when only ultrasound was applied or a ratio of 2.03 when only AuPEG coating was added.

Unfortunately, none of the published studies of the efficacy of Ad5-mir122 on HepG2 xenograft reported any statistical analysis of log-rank test result or hazard ratio, so no direct comparison could be made. However, these studies did indicate that only after day 20 and onward, the tumour volumes of mice treated with Ad5-mir122 were significantly lower than mice treated with PBS [45]. In addition, even though a few published papers did show

ultrasound-enhanced virotherapy of un-modified Ad5 and polymer-coated Ad5 *in vitro* and *in vivo* for bio-distribution [45, 261], only one efficacy paper on ultrasound-enhanced virotherapy was published by Greco *et al.* However, this study by Greco and colleagues contained some flaws in experimental design and data analysis. In the study, two DU-145 tumour xenografts were grown on the left and right flanks of each nude mouse. After administering microbubbles containing Ad5 per mouse, one tumour was treated with ultrasound and the other was not ultrasound-treated. The authors claimed that ultrasound enhanced the treatment by reduction in tumour volume over the course of 15 weeks; however, the tumour data showed that both the ultrasound-treated group and the non-treated group grew and shrank at a similar rate, suggesting it was virotherapy that caused the decrease in tumour size. In addition, the paper lacked the essential control group of PBS-treated mice [212].

5.3.7 Results: quantification of Ad5 in tumour at cull

Immediately after each mouse was culled (at appropriate but different time points), their tumours were extracted, homogenised, and purified for viral DNA. qPCR was used to quantify the total amount of replicative Ad5 inside each tumour, as previously described in section 3.3.1. In the Ad5–AuPEG groups, this enables quantification of the amount of virus that had been successfully cleaved and was therefore active. The average Ad5 particles detected in the tumours of the five treatment groups were shown in **Figure 5.17**.

The initial dose administered per mouse was 10^{10} copies of viruses. Recalling the analysis made in section 5.3.5 for **Figure 5.4** which reported the v.p. accumulation percentage per gram of tumour mass, the actual v.p. accumulation in the smaller initial volumes of tumours for the survival study could be estimated, along with ideal 42% (*in vitro* data from Chapter 4) of the total delivered Ad5–AuPEG were reactivated. As a result, the ‘working’ dose percentages for Ad5, Ad5 + US, Ad5–AuPEG, and Ad5–AuPEG + US were initially

0.12%, 0.69%, 0.084%, and 1.2%. With this initial tumour delivered dose established and by combining data shown in **Figure 5.17**, the replication of Ad5, Ad5 + US, Ad5–AuPEG, and Ad5–AuPEG + US resulted in 741-fold, 214-fold, 387-fold, and 3039-fold increases in intratumoural number of viral particles following culling of the mice.

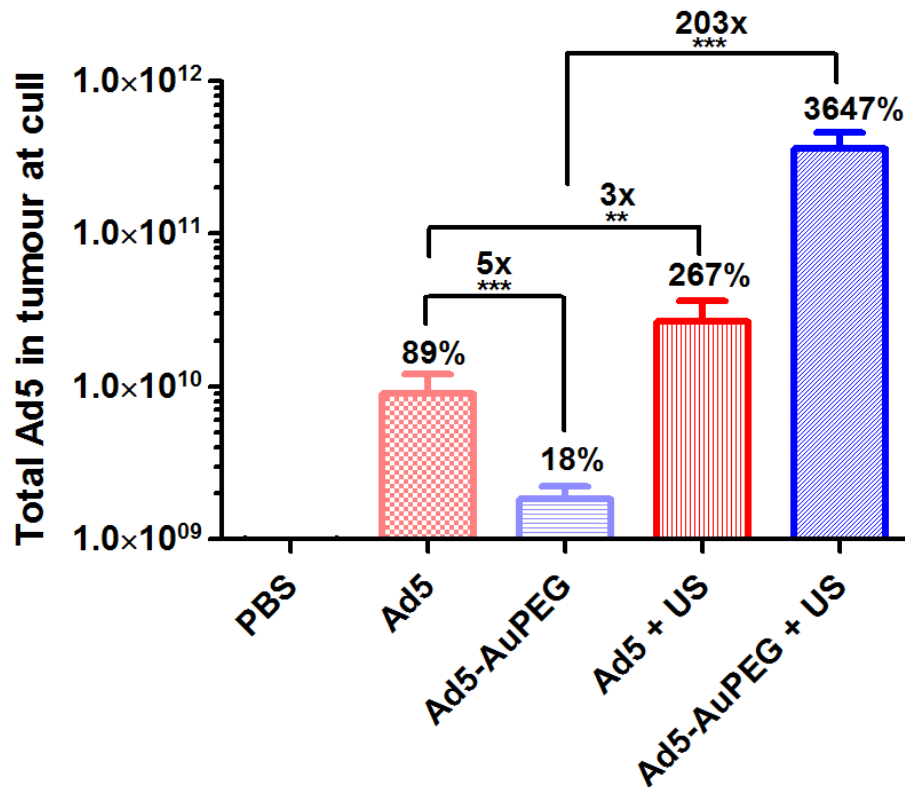


Figure 5.17: Total Ad5 particles detected in tumours at cull. After tumour tissues were homogenised, qPCR was performed to calculate the total amount of v.p. in all tumours. Each group had eight mice ($n = 8$), S.D. shown. Groups compared using one-way ANOVA followed by a Newman-Keuls test for pairwise comparison of sub-groups; ** and *** represent p -value < 0.01 , and 0.001 , respectively.

The number of v.p. detected in tumours from Ad5 + US was initially 7-fold greater than Ad5 ($p < 0.001$; **Figure 5.9**), but at the time of the cull, the former was only 3-fold more ($p < 0.01$; **Figure 5.17**); a possible explanation was that although ultrasound did extravasate more Ad5 into the overall tumour volume, some of the Ad5 was delivered to necrotic regions, resulting in low replication of the Ad5. On the other hand, the ratio of Ad5–AuPEG + US to Ad5–AuPEG delivered was 14, yet at the time of the cull, this ratio increased to 203, showing that the exponential replication of Ad5 was a powerful tool to suppress tumour growth as seen earlier in section 5.3.5. This may suggest that the Ad5–AuPEG delivered to perivascular

regions was relatively less active than the Ad5-AuPEG driven deeper into the tumour by ultrasound, in accordance with increased reducing potential and virus reactivation deeper into the tumour. It was important to note that although the label ‘Ad5–AuPEG’ was used to denote the initial treatment condition, once Ad5–AuPEG entered the HepG2 host cells and replicated, all resulting products were simply Ad5 only.

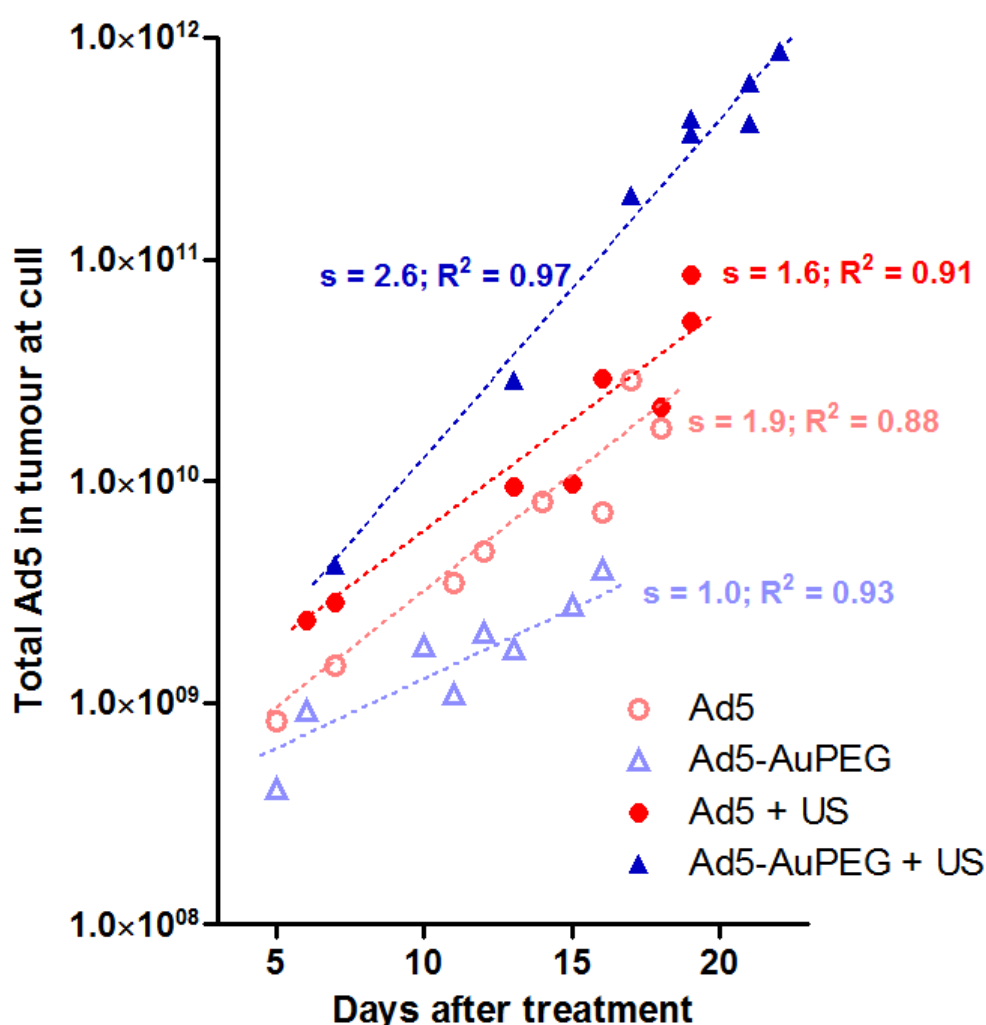


Figure 5.18: Total Ad5 particles detected in individual mouse tumour as a function of culling time. The horizontal axis show the exact day when individual mice were culled, and the vertical axis showed corresponding total copy of Ad5 detected by qPCR. The linear regression lines of ‘best fit’ were drawn for the four treatment group. R^2 is the coefficient of determination, which describes how well the data points fit the regression line; s = slope of the four regression lines, which were normalised to Ad5–AuPEG.

Although **Figure 5.17** only shows the overall average quantification of v.p. in tumour at the time of cull, each mouse was culled at different time points. To better describe how Ad5 replicated throughout the duration of survival study, **Figure 5.18** shows individual m total

tumour Ad5 content for individual mice plotted against the day it was culled. The linear regression lines of ‘best fit’ were generated for all four treatment groups, and their slopes (s) and R^2 are reported. All the slopes were normalised to the values for Ad5–AuPEG on each day, which were defined as 1. The values of R^2 were in the range of 0.88 to 0.97, showing that these regression lines were a very good fit to describe the trend of the data points drawn.

The steeper the slope, the faster the replication and growth rate of the virus. With a relative slope of 1, Ad5–AuPEG had the slowest rate of virus replication, and Ad5–AuPEG + US had the steepest relative slope of 2.6 with highest replication rate. The relative slopes for Ad5 and Ad5 + US were 1.9 and 1.6, respectively, showing that Ad5 actually had a slightly higher replication rate than Ad5 + US. This may explain the previous observation that a 7-fold increase in Ad5 + US vs Ad5 at day 0 yielded only 3-fold increase on the day of cull.

5.4 Summary and discussion

This chapter first investigated the plasma circulation, liver capture, and tumour accumulation of un-modified Ad5, two well established Ad5 therapeutics (Ad5–PEG and Ad5–PHPMA), and the newly proposed Ad5–AuPEG. Sections 5.2.2 to 5.2.4 not only reported the findings that Ad5–AuPEG was the most promising candidate suitable for ultrasound-enhanced delivery *in vivo* but also showed that the pharmacokinetic data gathered from Ad5, Ad5–PEG, and Ad5–PHPMA were comparable to previously published literature [88, 97, 119]. These validations reinforced that these data were reproducible and also strengthened the argument that Ad5–AuPEG was worthy of further *in vivo* study. This is the first time that a direct comparison of Ad5–PEG and Ad5–PHPMA has been made, and this study concluded that Ad5–PHPMA was the better candidate in terms of extended plasma circulation and reduced liver capture.

The *in vivo* work involved two mouse tumour models grown subcutaneously using two different cell lines. The model of BALB/c mouse with CT26 cells was useful in testing

pharmacokinetics, and the CD1 mouse model with HepG2 cells provided an efficient way to study efficacy of Ad5–AuPEG with therapeutic ultrasound.

The ultrasound exposure showed safe operation (including pressure, duty cycle, frequency, and microbubble concentration) to instigate the maximum cavitation activity from SonoVue® microbubbles. The analysis of the cavitation data by PCD revealed several characteristics: the sudden rise in broadband emissions upon re-injection of SonoVue® and the persistence of inertial cavitation over time; since our Ad5 therapeutics were co-injected with SonoVue®, these findings confirm the success of injection and treatment.

Currently there is no government-approved oral or intravenously administration of clodronate liposome. Translating present *in vivo* works into clinical practice by the administering of clodronate liposome is a challenge to overcome. In the absence of clodronate liposome administration, Ad5–PHPMA (not Ad5–AuPEG) was actually the leading therapeutic agent which circulating the longest in bloodstream and accumulate the least in liver. However, *in vitro* study on OptiCell® phantom (Chapter 4) showed that Ad5–PHPMA extravasated at similar level as un-modified Ad5 under the initial cavitation regime. As a result, even without the treatment of clodronate liposome, Ad5–AuPEG (second best circulating Ad5) could still potentially enter clinically trial with therapeutic ultrasound to provide an enhanced delivery to solid tumour in cancer patients.

According to the official ‘SonoVue Summary of Clinical Safety Experience’ on SonoVue® microbubbles filed by Bracco Diagnostics Inc. and approved by the FDA in 2011, the current clinically approved administration of SonoVue® is 2 mL per injection and a maximum of two injections per patient per visit [284]. The average weight of a human is 70 kg [285]; this means that the maximum 4-mL recommend dose of SonoVue® per visit translates into 57 µL of Sonovue® per kg of human weight. However, a study by Bokor *et al.* provides clinical evidence that SonoVue® doses of up to 120 µL/kg are safe [286].

All the mice used in this *in vivo* study had mature body weights in the range of 25 g to 28 g and received 150 μL of SonoVue® in multiple injections but over the course of a single treatment. This corresponds to a dose of approximately 5300 $\mu\text{L}/\text{kg}$, which is 44 times larger than the maximum dose shown to be safe in humans. It should be noted that this disparity in SonoVue® dose can be countered by the disparity in tumour to body weight ratio between humans and murine models. A 50-mm³ mouse tumour (the volume used when the treatment was given in this survival study) will typically have a mass of 0.05g, which represents some 0.2% of the body weight of the mouse. The corresponding tumour weight in a 70-kg human would be 140 g, which is equivalent a 140-cm³ spherical tumour with a diameter of 6.5 cm, yet studies have shown that majority of pharmacologically treated patients have detectable tumours with diameter less than 1 cm [287]. The volume ratio of the equivalent 140-cm³ human tumour to the 50-mm³ mouse tumour is therefore 2800. A higher relative dose of SonoVue® does therefore need to be delivered in mice to ensure comparable perfusion of tumour tissue in this study.

Despite the fact that the total 150 μL of SonoVue® injected per mouse in this study exceeded the clinically approved limit, the main focus of the *in vivo* study was to test ultrasound-enhanced extravasation in solid tumour. This emphasises a limitation of the current approach in that a constant supply of gas bubbles is needed to provide nuclei for the initiation of inertial cavitation. As the process is destructive and the gas bubbles have a short half-life in circulation, this presents an important barrier to clinical utility. Despite these issues is important to note that no adverse side effects upon SonoVue® administration were observed in these studies or in previous studies by Bazan-Peregrino *et al.* Indeed, over the course of the entire survival study, all the mice were motile, active and sociable and demonstrated no level of pain or distress, suggesting the SonoVue® dose administered in this survival study caused no long-term harm to the mice. Before translating this work into clinical practice, the optimal concentration of SonoVue® microbubbles would need to be re-evaluated

since SonoVue® was manufactured for the purpose of imaging instead of becoming cavitation nuclei.

The HepG2 tumour growth rate in this study was increased compared to other reported HepG2 xenograft growth rates [274, 282]. If this study were to be repeated, then a less aggressive HepG2 or other liver carcinoma would be utilised. However, these studies had the ultimate goal of specifically investigating the role of ultrasound in enhancing the delivery and anti-tumour efficacy of both virus and virus engineered for ultrasound-enhanced delivery. Therefore, unlike in most virotherapy studies where multiple doses are given at different time points [97, 274], a single dose administration of Ad5 was performed here to enable the added value of ultrasound treatment to be investigated. If multiple and higher doses of Ad5 were administered, discerning the effect of the ultrasound assisted delivery from the intrinsic activity of the virus may be difficult. However, it is notable that multiple high dose regimes may ultimately prove to be clinically unacceptable and/or wasteful as immune responses raised against the first injection of Ad5 may dramatically limit the efficacy of further doses. Approaches such as the one described here to improve the tumour accumulation and activity of the dose that is delivered may be a more realistic approach than simply administering more Ad5 more often.

Despite not seeing strong luciferase signals of virus replication from the IVIS imaging, this *in vivo* study was the first to quantify the total number of Ad5 inside the tumours at cull. All other published studies rely solely on the luciferase signals to determine virus infection of and replication in tumours; however, with only 10% of the initial Ad5 dose encoding luciferase in this study, qPCR assessment was a better measurement to take into account both Ad5-mir122 and Ad5-mir122-luc replication. **Figure 5.18** was also the first graph of its kind to analyse the virus replication rate by plotting the days of mouse cull against the tumour Ad5 load. This new way of analysing the virus replication data is recommended for future studies.

CHAPTER 6

CONCLUSION AND FUTURE WORK

6. Conclusions and future work

6.1 Conclusions

In addition to being safe, inexpensive and minimally invasive, the exploitation of inertial cavitation for ultrasound-enhanced drug delivery presents another major advantage which very few modalities can offer. Specifically, cavitation events can be detected remotely, and can therefore act not only as a promoter but also as a marker of successful drug delivery. The recent development of a novel technique known as passive acoustic mapping [137, 138], provides a unique opportunity for spatio-temporal mapping of cavitation activity using conventional ultrasound imagers, and could thus also provide a method for informing clinicians of when and where a therapeutic agent has been successfully delivered.

The main objective of this thesis was to develop a better platform to optimise ultrasound-enhanced virotherapy to solid tumours. In Chapter 1, fundamental principles of tumour development were first discussed, followed by an extensive review of the current limitations of oncolytic adenovirus and its recent development for ‘stealthiness’ by chemical modifications (PEG and PHPMA). The literature pertaining to therapeutic ultrasound was surveyed, and various ultrasound-enhanced delivery vehicles and encapsulated nanomedicines were reviewed. The high safety standard for both virotherapy and therapeutic ultrasound was emphasised. The conclusion for this literature review was that the integration of oncolytic adenovirus with therapeutic ultrasound had tremendous potential but that significant improvements needed to be made, particularly in terms of circulation kinetics and delivery.

The concept of coating Ad5 with PEGylated gold nanoparticles (AuPEG) via reducible disulphide bond was introduced in Chapter 2. The chemical modifications of Ad5–AuPEG were reported, and the characterisation of Ad5–AuPEG was performed using DLS, ζ -potential, SDS-PAGE silver staining, TEM, TNBS assay, and CsCl density-gradient

centrifugation. Together, these six different tools confirmed the successful synthesis of Ad5–AuPEG: DLS, zeta potential, SDS-PAGE, ELISA, TEM, and TNBS. The newly synthesised Ad5–AuPEG underwent a similar increase in size compared with Ad5–PEG and Ad5–PHPMA both as co-formulated here and as reported in the literature [103, 117], yet the ζ -potentials were very different among all three Ad5 modifications, suggesting successful coating of Ad5 by these three different strategies.

The *in vitro* studies of Ad5–AuPEG are presented in Chapter 3. In particular, its protection against neutralisation by antibodies, infectivity in low-CAR and high-CAR cells, reduction efficiency and cleavage of the disulphide bond between Ad5 and Au, and binding to human erythrocytes were tested. Ad5–AuPEG showed a significant reduction in anti-Ad5 antibody neutralisation, a total knockdown of both IGROV-1 and SKOV-3 infection, and a 10-fold decrease in human erythrocyte binding. Whilst the IGROV-1 infection assay suggested that the infection of Ad5–AuPEG could be ‘switched on’ under tumour-like pH and reducing environment, the SKOV-3 infection assay demonstrated hexon regions of Ad5–AuPEG were effectively stealthed since the addition of FBS did not increase the infection of these low-CAR cells. Ad5–AuPEG showed superior reduction in antibody binding (90% reduction) compared to Ad5–PEG (10% reduction) and Ad5–PHPMA (75% reduction).

Building on previous studies which had optimised focussed ultrasound parameters at 0.5 MHz to enhance virus extravasation, a tissue-mimicking flow channel phantom model was developed in Chapter 4 to examine the hypothesis that increasing the density of Ad5 could increase its distribution into the phantom. Sonovue microbubbles were introduced as exogenous cavitation nuclei by co-delivery with Ad5, Ad5–PHPMA, or Ad5–AuPEG. Passive cavitation detection (PCD) was used to detect cavitation activity by recording the acoustic emissions generated during microbubble oscillations. PCD results showed ultraharmonic energy at 180 kPa corresponding to stable cavitation and broadband energy at 625 kPa and 1250 kPa corresponding to inertial cavitation. The results showed that the larger

the ultrasound exposure pressure, the more cavitation activity was detected, suggesting a concordant increase in the energy delivered. The transport of Ad5–AuPEG was enhanced by two orders of magnitude by 1250 kPa (peak negative pressure), yet the same ultrasound exposure was only able to enhance the extravasation of both Ad5 and Ad5–PHPMA by an order of magnitude. These results showed for the first time that denser Ad5 had the tendency to be ‘pushed’ more efficiently in terms of both furthest distance travelled and total number of v.p. delivered. A proposed hypothesis was that all modified and un-modified Ad5 had reached their respective terminal velocities after receiving the energy from the collapsing of microbubble cavitation nuclei; since momentum is directly related to mass and velocity, the heaviest Ad5–AuPEG possessed the greatest momentum to extravasate further in these tumour-mimicking phantom models.

Such *in vitro* data provided a compelling case for further *in vivo* investigations. Chapter 5 presents the *in vivo* study in three phases: phase one describes the plasma circulation and bio-distribution of Ad5, Ad5–PEG, Ad5–PHPMA, or Ad5–AuPEG in BABL/c mice bearing CT26 tumour; phase two describes the influence of ultrasound/Sonovue induced cavitation on the tumour accumulation of Ad5–AuPEG compared to Ad5; phase three characterises the influence of ultrasound-enhanced virus delivery on treatment efficacy and survival rate of CD1 mice bearing HepG2 xenografts. Phase one demonstrated that 30 min after v.p. administration into clodronate-treated mice, Ad5–AuPEG had lower liver capture than Ad5 ($p < 0.001$), Ad5–PEG ($p < 0.001$), and Ad5–PHPMA ($p < 0.05$). This provided a higher level of plasma circulation than Ad5 ($p < 0.001$), Ad5–PEG ($p < 0.001$), and Ad5–PHPMA ($p < 0.001$), and enhanced tumour accumulation compared to Ad5 ($p < 0.001$), Ad5–PEG ($p < 0.001$), and Ad5–PHPMA ($p < 0.01$). In addition, for the first time, a direct comparison between Ad5–PEG and Ad5–PHPMA was shown, demonstrating that Ad5–PHPMA has significantly ($p < 0.001$) better pharmacokinetics than Ad5–PEG. Phase two results supported the *in vitro* work produced

using OptiCell® flow channels, confirming the hypothesis that higher-density Ad5–AuPEG extravasated more efficiently than Ad5 under inertial cavitation regime. In the presence of ultrasound exposure, 12% of the injected Ad5–AuPEG accumulated at tumour, compared to only 0.69% of the injected Ad5. This dramatically increased level of tumour accumulation has never been previously reported for oncolytic viruses. Phase three demonstrated that mice treated with Ad5–AuPEG + US had the slowest tumour growth, highest survival rate, and largest quantity of v.p. detected in tumours at cull. Interestingly, mice treated with Ad5–AuPEG alone showed no significant difference in reduction of tumour growth and survival rate compared to Ad5 despite a much higher initial dose accumulating within the tumour. This may be explained by a failure of the disulphide bonds linking Ad5 to AuPEG to cleave. In ultrasound treated mice such cleavage may have been assisted by deeper penetration of the Ad5–AuPEG into tumour region with higher reducing capacity [32].

In summary, the newly proposed ‘dandelion’ structure of Ad5–AuPEG provided very effective steric shielding with only minimal and reversible modification of the Ad5 capsid. However, in addition to providing a platform for effective stealthing, the Au nanoparticles also increased the density of Ad5. This thesis describes how this increase in density imparted a second major advantage on the strategy. Specifically, the response of Ad5 to ultrasound exposure is dramatically improved upon AuPEG addition, leading to a substantial efficacy enhancement.

6.2 Future work

The next challenge in ultrasound-enhanced drug delivery is to develop a new generation of delivery vehicles which are tailor-made for cavitation-enhanced drug delivery for cancer. Increasing bloodstream stability and pharmacokinetics and achieving co-location of the delivery vehicles with cavitation nuclei, are important goals. However, just improving the delivery vehicles alone is not sufficient.

Cancer cell-specific Ad5 can now be genetically engineered to provide enhanced specificity for tumours while avoiding viral infection to other healthy tissues. Given the many therapeutic systems already in clinical use [127, 228] and the approval by the FDA for several Ad5s to be used in Phase III clinical trials [218, 219], ultrasound-enhanced virotherapy may well be rapidly approaching clinical applicability. To translate this technology into clinical trials, more precise ultrasound exposure parameters must be defined, ideally with low pressure required for cavitation initiation. Given the current knowledge acquired from *in vitro* and *in vivo* studies, short ultrasound exposure times (less than a few minutes), frequencies between 0.5 MHz and 2.5 MHz and pressures up to 2.0 MPa are recommended for safe and effective ultrasound therapy.

There are many approaches to improve the current ultrasound-enhanced virotherapy presented in this thesis. Firstly, Au particles can be easily replaced with other heavy solid nanoparticles (e.g. silver, iron oxide, titanium oxide, quantum dots) to investigate the ideal density-augmentation strategy for Ad5 extravasation under safe therapeutic ultrasound exposure regimes. This study has proposed and confirmed that higher-density Ad5–AuPEG was more effectively extravasated than Ad5, yet it is unclear if there is an optimal density needed for Ad5-modified therapeutics. By replacing the Au used in this study with other clinically approved metal nanoparticles, there is a great opportunity to enhance the extravasation of coated Ad5 still further or to extravasate by the same distance but lower threshold of ultrasound pressure.

Secondly, the design of ‘dandelion’ structure can be applied universally not just for coating Ad5, but also for other nano-medicines (e.g. liposomes) to give a small percentage of surface modification yet effective overall shielding.

Thirdly, instead of using IGROV-1 and SKOV-3 cells for high and low expression of CAR, respectively, a better pair of cell lines to test the cell infection assays (described in

Chapter 3) is CHO and CHO-CAR cells. CHO stands for Chinese hamster ovary cell. While CHO cells have been genetically engineered to completely delete its membrane CAR expression, CHO-CAR cells have been regulated to express higher level of CAR. Since both CHO and CHO-CAR were derived from the same origin, repeating the assay on how they influence Ad5 infection via the CAR-binding motif will give a more direct comparison.

Fourthly, to test the true effect of FX-mediated binding of Ad5, FX at physiologically relevant concentration (10 µg/mL) should be added to serum-free cell medium. Ad5 and Ad5–AuPEG can then infect CHO cells at serum-free cell medium or cell medium with FX to determine the true identity of FX ‘bridging’ mechanism between Ad5 and CHO cells.

Fifthly, for the OptiCell® experiment, a replicative Ad5 could be used to trace the spread of the viral infection in OptiCell® phantom models over time. This would allow a better visual understanding of how Ad5 infect its neighbouring cells and its being monitored in real time.

Sixthly, different ultrasound parameters (e.g. frequency, pressure, PRF, duty cycle) could be optimised to ensure the maximum perfusion of UCAs at different tumour volumes. Although the growth of smaller tumours (size < 50 mm³) is more easy to suppress, they often lack an ‘angiogenesis switch’ and have weaker EPR effect leading to lower perfusion and recruitment of UCAs and Ad5. Larger tumours (size > 150 mm³) have undergone the ‘angiogenesis switch’ so have improved accumulation of UCAs and Ad5, but are usually in exponential growth phase and very hard to suppress by oncolytic viruses or any other agent (as seen in Chapter 5 of this study). Two sets of ultrasound exposure parameters should be defined: one for small tumours and another one for large tumours. This is extremely important for clinical translation, as cancer patients often seen tumours with various sizes when detected.

Seventhly, for all future works, all major organs from animal models should be extracted to quantify Ad5 accumulation. In addition to the murine organs extracted in this DPhil study (e.g. tumour, liver, spleen, and blood), heart, lung, brain, kidney, stomach, and intestine should also be extracted for bio-distribution analysis. This will allow the complete understanding of different modified Ad5 accumulation to better design future virotherapy for specific target organs.

Eighthly, to improve the low bioluminescence intensities detected in the IVIS imaging from only 10% of the injected Ad5 carried luciferase gene, future survival study should use 100% Ad5 that express luciferase gene. This will increase the bioluminescence intensity, so the IVIS imaging could then trace the replication of Ad5 overtime in both tumour and other organs.

Ninthly, perhaps another human liver carcinoma cell line (or different batch of HepG2 with known slower growth rate) could be used to give Ad5-mir122 more time to replicate and eventually suppress the tumour. A small pilot study could be conducted first by simply implanting several liver carcinomas on mouse models and observing the tumour growth rate; the liver cell line with the slowest growth rate can then be used subsequently for Ad5-mir122 survival study to demonstrate an even more significant improvement on the efficacy study.

Lastly, most cavitation nuclei used to date have been micron-sized UCAs which are confined to the vascular space and cannot extravasate into tumours via the EPR effect. A new generation of sonosensitive nanoparticles were recently developed in Oxford which would make it possible to overcome this limitation. These nanoparticles entrap nano-size air bubbles on their surface which survive repeated ultrasound exposure, and have been shown to generate comparable levels of cavitation energy to UCAs with reduced requirement for constant replenishment [192, 288]. These solid nanoparticles could thus be linked directly to Ad5 to ensure co-localisation of the virus and cavitation nuclei beyond the vasculature.

However, the overall size needs to be maintained below 500 nm, allowing optimal extravasation into the tumour interstitial space. Another alternative is that researchers can ideally produce ‘real’ acoustically active nanobubbles that show a mono-disperse size distribution. However, to date, there is an absence of compelling evidence that ultrasound-responsive bubbles that are truly nano-scale have been formulated, as reviewed in Chapter 1. The invention of sonosensitive solid nanoparticles is a promising approach, and more studies are needed to validate and understand the mechanisms of these ‘semi-indestructible’ UCAs.

CHAPTER 7

APPENDIX

7. Appendix

7.1 Peer-reviewed journal articles

- **S. Mo**, R. Carlisle, R. Laga, R. Myers, S. Graham, R. Cawood, K. Ulbrich, L. Seymour, and C.-C. Coussios. "Ultrasound-enhanced virotherapy by increasing viral density with gold nanoparticles." submitted to *Nature Nanotechnology* (in review)
- **S. Mo**, C.-C. Coussios, L. Seymour, and R. Carlisle. "Ultrasound-enhanced drug delivery for cancer." *Expert Opinion on Drug Delivery* **9**(12): 15125-1538 (December 2012).
- S. Nandlall, M. Bazan-Peregrino, **S. Mo**, and C.-C. Coussios. "Determination of Cytotoxic Thermal Dose during HIFU Ablation." Proceedings of the 11th International Symposium on Therapeutic Ultrasound, *AIP Conference Proceedings* **1481**: 259-264 (April 2011).

7.2 Peer-reviewed abstracts

- R.C. Carlisle, **S. Mo**, S. Graham, R. Myers, E. Mylonopoulou, L. Seymour, C.-C. Coussios. "Increased adenovirus density for increased ultrasound-mediated delivery to tumours." *2013 European Society of Gene and Cell Therapy*; Madrid, Spain on 25-28 October, 2013.
- **S. Mo**, R. Myers, C.-C. Coussios, R.C. Carlisle, L. Seymour. "Gold nanoparticles to enhance stealthing of adenovirus." *2013 European Society of Gene and Cell Therapy*; Madrid, Spain on 25-28 October, 2013.
- **S. Mo**, C.-C. Coussios, L. Seymour, and R.C. Carlisle. "Coating of adenovirus type 5 with cleavable PEGylated gold nanoparticles for enhanced protection and circulation." *10th annual conference of the British Society for Gene and Cell Therapy*; London, UK on 17-19 April, 2013.

7.3 Conferences attended

- *2012 Annual Meeting of the Wellcome Trust/EPSRC Medical Engineering Centres*. Oxford, UK on 4-5 September, 2011
- *12th International Symposium on Therapeutic Ultrasound*. Heidelberg, Germany on 10-13 June, 2012.
- *2011 Annual Meeting of the Wellcome Trust/EPSRC Medical Engineering Centres*. York, UK on 25-26 May, 2011

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