OPTIMISING POLYMERSOMES FOR IMAGING TUMOUR AND ITS ENVIRONMENT

Adrian S. Joseph

Thesis submitted for the Degree of Doctor of Philosophy

Department of Oncology, University of Sheffield

2014
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July 2014
DECLARATION

I hereby declare that no part of this thesis has previously been submitted for any degree or qualification of this, or any other University or Institute of learning
ACKNOWLEDGEMENTS

First and foremost I want to thank my supervisors, Prof. Nicola Brown and prof. Giuseppe Battaglia. This work could have not been completed without their help and guidance. All their contributions and ideas helped me to complete this work. I am also extremely grateful to them for all the suggestions and corrections in the long writing stage.

I would also like to thank Cancer Research UK, Engineering and Physical Sciences Research Council and the Department of Health for the funding this work, and the PI of the project Prof. G. Tozer and Prof. M. Paley for their advices on the general direction of the work.

The completion of this multidisciplinary work was made possible only by the contribution of many people. I am grateful to Dr. Nick Warren and Dr. Jeppe Madsen from Prof. Steve Armes group for synthesising the co-polymers. I am also thankful to James Robertson and Dr. Denis Cecchin for their advices on polymersomes synthesis and characterisation and to Russell Pearson, Claudia Contini and Chris Hill for the help with transmission electron microscopy sample preparation and imaging. Coming from a biological background, none of the flow *in silico* simulations would have been possible without the precious help of Dr. Carla Pegoraro, who introduced me to the usage of Comsol Multiphysics, a key tool in the development of the flow bioincubator.

I am sincerely grateful to Matthew Fisher and Dr. Sarah-Jane Lunt for all the long hours spent in the filed lab during the *in vivo* work. Without their help it would have not been possible to complete any of the surgeries. I am also thankful to Colin Gray, whose help in setting up the microscope for intra-vital microscopy was crucial, and to Dr. Chryso Kanthou and Dr. Joanne Bluff for the advices with 10T1/2 and mouse fibrosarcoma cells culturing.

Completing this work was not only about experimental work. The process of writing, editing and correction of the manuscript was challenging, and I would like to thank again my supervisors Prof. Brown and Prof. Battaglia for their guidance. Furthermore, I am grateful to Gavin Fullstone and Dr. Claire Bennison, who kindly proofread part of the manuscript.

A sincere thank you to family and friends. They all have been supportive and understanding during the most difficult times, giving me the much needed extra strength to renew motivation and to replenish energies to continue with the work.

There is a long list of people who helped me during this time, both inside and outside the lab, just too long to be listed one by one. Even if you do not find your name listed above, to all of you goes my sincere thank you.
Polymersomes are nano-meter sized vesicles made by the self assembly of amphiphilic co-polymers in water. This work presents the development of polymersomes as a tool for high resolution in vivo optical imaging. High resolution Intra Vital Microscopy (IVM) was made possible by the use of the Window Chamber (WC), a surgical procedure which allows observation of living tissues through an optically transparent glass replacing a section of skin. WC can be used to observe tumour growth and tumour vasculature development (angiogenesis). High resolution imaging of angiogenesis in vivo with polymersomes can give an useful insight in mechanism of angiogenesis, tumour response to anti-angiogenic therapy and the rationale behind the design of polymersomes for specific tumour targeting in therapy.

In this work we optimised a number of techniques and tools to characterise the interactions of two different polymersome formulation with three cell types relevant to the tumour microenvironment: tumour cells Mouse Fibrosarcoma Cell (MFC), endothelial cells Small Vessel Endothelial Cells (SVECs) and perivascular-like cells 10T1/2. The techniques used included Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC), Fluorescence Activated Cell Sorting (FACS) and optical imaging. Furthermore, a flow bio-incubator was developed to study the effect of physiologically relevant flow rates on cellular polymersome uptake. Finally, formulations were tested in vivo to assess suitability for optical imaging and polymersomes distribution in tumour vasculature and stroma. Specific image analysis tool were developed to assist the analysis.

The results demonstrated that polymersomes can be used for optical imaging in vivo using a WC. The in vitro techniques developed allowed us to quantify the interactions between polymersomes and cells, and the information gained can be translated in vivo to help predict the polymersomes distribution in tumour.

Taken together, the methods and results presented here provide a set of tools based on image analysis that allow rational design of polymersomes for in vivo application.
PUBLISHED ABSTRACTS


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<th>Notation</th>
<th>Description</th>
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<tbody>
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<td>α-SMA</td>
<td>α-Smooth Muscle Actin.</td>
</tr>
<tr>
<td>ADME</td>
<td>Administration, Distribution, Metabolism, Excretion.</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy.</td>
</tr>
<tr>
<td>ALK-1</td>
<td>Activin receptor-like kinase 1.</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection.</td>
</tr>
<tr>
<td>ATRP</td>
<td>Atom Transfer Radical Polymerisation.</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay.</td>
</tr>
<tr>
<td>BE</td>
<td>Binding efficiency.</td>
</tr>
<tr>
<td>BME</td>
<td>Basal medium eagle.</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumine.</td>
</tr>
<tr>
<td>CAC</td>
<td>Critical Aggregation Concentration.</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer Associated Fibroblast.</td>
</tr>
<tr>
<td>CFD</td>
<td>Computational fluid dynamic.</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal Laser Scanning Microscopy.</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System.</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography.</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering.</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium.</td>
</tr>
<tr>
<td>DoG</td>
<td>Difference of gaussians.</td>
</tr>
<tr>
<td>DPA</td>
<td>2-(diisopropylamino)ethyl methacrylate.</td>
</tr>
<tr>
<td>DSC</td>
<td>Dorsal Skinfold Chamber.</td>
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<td>Notation</td>
<td>Description</td>
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<tr>
<td>EC</td>
<td>Endothelial cell.</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence.</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix.</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor.</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor.</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition.</td>
</tr>
<tr>
<td>EndT</td>
<td>Endothelial-Mesenchymal Transition.</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced Permeation and Retention.</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting.</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum.</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drugs Administration.</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor.</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein.</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel Permeation Chromatography.</td>
</tr>
<tr>
<td>GUI</td>
<td>Graphic user interface.</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid.</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible Factor-1α.</td>
</tr>
<tr>
<td>HMWMMAA</td>
<td>High Molecular Weight Melanoma Associated Antigen.</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intra Muscular.</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intra Peritoneal.</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intra Venously.</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intra Venous.</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry.</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin of the class G.</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry.</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial Methylated Spirit.</td>
</tr>
<tr>
<td>IVM</td>
<td>Intra Vital Microscopy.</td>
</tr>
<tr>
<td>LTE</td>
<td>Local threshold entropy.</td>
</tr>
<tr>
<td>MFC</td>
<td>Mouse Fibrosarcoma Cell.</td>
</tr>
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<td>Notation</td>
<td>Description</td>
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</tr>
<tr>
<td><strong>MFC120</strong></td>
<td>Mouse Fibrosarcoma Cells expressing VEGF120 isoform.</td>
</tr>
<tr>
<td><strong>MFC188</strong></td>
<td>Mouse Fibrosarcoma Cells expressing VEGF188 isoform.</td>
</tr>
<tr>
<td><strong>MPC</strong></td>
<td>2-methacryloxyethyl-phosphorylcholine.</td>
</tr>
<tr>
<td><strong>MPM</strong></td>
<td>Multi Photon Microscopy.</td>
</tr>
<tr>
<td><strong>MPS</strong></td>
<td>Mononuclear Phagocyte System.</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td>Magnetic Resonance Imaging.</td>
</tr>
<tr>
<td><strong>MSC</strong></td>
<td>Mesenchymal Stem Cells.</td>
</tr>
<tr>
<td><strong>MTT</strong></td>
<td>(3 - (4,5 - Dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide.</td>
</tr>
<tr>
<td><strong>MVD</strong></td>
<td>Micro Vessel Density.</td>
</tr>
<tr>
<td><strong>MW</strong></td>
<td>Molecular weight.</td>
</tr>
<tr>
<td><strong>MWP</strong></td>
<td>Micro well plate.</td>
</tr>
<tr>
<td><strong>NaOH</strong></td>
<td>Sodium Hydroxide.</td>
</tr>
<tr>
<td><strong>NG2</strong></td>
<td>Nerve Glial antigen 2.</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>Phosphate Buffer Saline.</td>
</tr>
<tr>
<td><strong>PBS_2</strong></td>
<td>Phosphate Buffer Saline pH 2.</td>
</tr>
<tr>
<td><strong>PBS_7.4</strong></td>
<td>Phosphate Buffer Saline pH 7.4.</td>
</tr>
<tr>
<td><strong>PDB</strong></td>
<td>Protein Data Base.</td>
</tr>
<tr>
<td><strong>PDGF</strong></td>
<td>Platelet Derived Growth Factor.</td>
</tr>
<tr>
<td><strong>PDGF-AA</strong></td>
<td>Platelet Derived Growth Factor-AA.</td>
</tr>
<tr>
<td><strong>PDGF-B</strong></td>
<td>Platelet Derived Growth Factor-B.</td>
</tr>
<tr>
<td><strong>PDGF-R_α</strong></td>
<td>Platelet Derived Growth Factor Receptor-α.</td>
</tr>
<tr>
<td><strong>PDGF-R_β</strong></td>
<td>Platelet Derived Growth Factor Receptor-β.</td>
</tr>
<tr>
<td><strong>PDPA</strong></td>
<td>poly(2-(diisopropylamino)ethyl methacrylate).</td>
</tr>
<tr>
<td><strong>PEO</strong></td>
<td>Polyethylene Oxide.</td>
</tr>
<tr>
<td><strong>PEO-PDPA</strong></td>
<td>Oxide-poly(2-(diisopropylamino)ethyl methacrylate).</td>
</tr>
<tr>
<td><strong>PET</strong></td>
<td>Positron Emission Tomography.</td>
</tr>
<tr>
<td><strong>PMPC</strong></td>
<td>Poly(2 - methacryloxyethyl - phosphorylcholine).</td>
</tr>
<tr>
<td>Notation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PMPC-PDPA</td>
<td>Poly(2-methacryloxyethylphosphorylcholine)-poly(2-(diisopropylamino)ethyl methacrylate).</td>
</tr>
<tr>
<td>POEGMA</td>
<td>poly(oligo(ethylene glycol) methacrylate).</td>
</tr>
<tr>
<td>POEGMA-PDPA</td>
<td>poly(oligo(ethylene glycol) methacrylate)-poly(2-(diisopropylamino)ethyl methacrylate).</td>
</tr>
<tr>
<td>PTA</td>
<td>Phosphotungstenic acid.</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride.</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial System.</td>
</tr>
<tr>
<td>RGS5</td>
<td>Regulator of G-protein Signalling 5.</td>
</tr>
<tr>
<td>ROI</td>
<td>Regions of interest.</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse Phase-High Pressure Liquid Chromatography.</td>
</tr>
<tr>
<td>s.c.</td>
<td>Sub Cutaneous.</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy.</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells.</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated Emission Depletion.</td>
</tr>
<tr>
<td>STORM</td>
<td>Stochastic Optical Reconstruction Microscopy.</td>
</tr>
<tr>
<td>SVECs</td>
<td>Small Vessel Endothelial Cells.</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour Associated Macrophages.</td>
</tr>
<tr>
<td>TE</td>
<td>Tripsin/EDTA.</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy.</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid.</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor-β1.</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran.</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour Infiltrated Lymphocytes.</td>
</tr>
<tr>
<td>USPIO</td>
<td>Ultrasmall Superparamagnetic Iron Oxide nanoparticles.</td>
</tr>
<tr>
<td>VDA</td>
<td>Vascular Disrupting Agent.</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor.</td>
</tr>
<tr>
<td>Notation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>VEGF120</td>
<td>Vascular Endothelial Growth Factor isoform 120.</td>
</tr>
<tr>
<td>VEGF164</td>
<td>Vascular Endothelial Growth Factor isoform 164.</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor.</td>
</tr>
<tr>
<td>VPF</td>
<td>Vascular Permeability Factor.</td>
</tr>
<tr>
<td>vSMCs</td>
<td>Vascular Smooth Muscle cells.</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot.</td>
</tr>
<tr>
<td>WC</td>
<td>Window Chamber.</td>
</tr>
</tbody>
</table>
1.1 Cancer. General characteristics

The term tumour describes a group of cells with abnormal and uncontrolled growth. As a tumour grows in size it compresses the surrounding normal tissue, but is localised to a specific area of the body at this stage. Once the tumour induces a vasculature it has the potential to escape the primary site and to induce secondary tumours.

Cancer is a high incidence disease which is characterised by high mortality: cancer was the second highest cause of death in UK in 2011 and it is estimated that one in three people will develop cancer at some time in their life (Sasieni et al. [2011]) (fig. 1.1). Furthermore, cancer accounted for the third highest expenditure by the NHS after mental disorders and cardiovascular diseases, totalling around £5.93bn, and the cost of cancer is estimated to raise to £15.3bn by 2021 (BUPA 2012 report). Thus, providing justification for additional research focusing on molecular, cellular and functional aspects to identify prognostic markers and effective treatment.

There are more than 200 different types of cancer, with names and characteristics reflecting specific localisation (i.e. glioblastoma, fibrosarcoma, melanoma, breast cancer etc.). Despite differences in tumour localisation and cell mutations there are morphological features common to almost all of the tumours. A solid tumour is characterised by the initial onset of cancer cells, defined as mutated cells, which grow and divide in an unregulated manner. Normal apoptotic mechanisms are not functional. The tumour microenvironment also consists of stroma cells including fibroblasts, immune cells, endothelial cells, and specialised mesenchymal cells, all playing an important role in the development of the primary tumour (Bremnes et al. [2011], Weinberg [2013]). Finally, tumours present a
1.2.1 Blood vessel anatomy

Blood has an essential role in the homoeostasis of our body as it distributes nutrients, oxygen, hormones and a number of other substances to every cell. Furthermore, blood collects metabolic waste such as carbon dioxide and other small substances and carries them to specialised organs (lungs, liver, kidney) for elimination. The exchange between nutrients and oxygen and waste occurs in the microcirculation, where the arterial system branches into smaller arterioles and finally capillaries (≈8μm diameter). The capillary bed has a much greater cross-section surface area and the blood flow is much slower when compared to bigger vessels (fig. 1.2). This facilitates the exchange of gases and nutrients between blood and extracellular matrix of the tissue fed by the capillaries.

The driving motor of the circulatory system is the heart. Major arteries and veins leave the heart and branch into smaller vessels and capillaries. Capillaries then rejoin into venules and veins. Main blood vessels and first branches are composed of three layers: tunica intima, tunica media and tunica adventitia, from the lumen to the outside. The tunica intima is composed of a layer of endothelial cells, the tunica media consists of elastic fibres and Vascular Smooth Muscle cells (vSMCs), especially in arteries. The tunica adventitia consists of connective tissue. Progressively smaller vessels have a much thinner wall and they are composed of a layer of endothelial cells surrounded by a basal lamina, supported by necrotic, hypoxic central region and are characterised by chaotic and unstable immature vasculature which results in continuous remodelling of the capillary bed in a process called angiogenesis.
a few perivascular cells. Capillaries in the brain, striated and smooth muscles, lung and connective tissues are known as continuous capillaries as they have no fenestration: endothelial cells form an uninterrupted wall. Other tissues such as kidneys, pancreas and endocrine glands have fenestrated capillaries with gaps of around 50nm-100nm. The gaps are covered with a matrix of unknown molecular composition that regulates substances trafficking (Standring [2008]).

Figure 1.2: Properties of the vascular system with respect to size. Capillaries have a higher surface area (green line) and lower flow velocity and pressure (red line) than arterioles and venules.

1.2.2 Mechanisms of angiogenesis

The capillary system is mainly formed de novo during the embryogenesis by a process known as vasculogenesis. The body has the ability to form specialised capillaries in different organs: capillaries in the liver are fenestrated and form a filtration system, in lungs sacks are formed to facilitate gas exchange. In adult life the formation of new blood vessels is limited to specific physiological situations such as menstrual cycle and wound healing. In addition, new capillaries are formed to meet specific requirements: for example, it has been reported that endurance athletes skeletal muscles have a more developed capillary bed compared to sedentary people (Carrow et al. [1967], Adolfsson et al. [1981], Coggan et al. [1992], Gielen et al. [2010]). In the human adult, new blood vessels are formed from pre-existing ones by a process known as angiogenesis. The ability to control vascular density based on supply/demand and the restoration of damaged vessels is the result of a delicate balance of factors promoting (pro-angiogenic factors) and inhibiting (anti-angiogenic factors) the formation of new blood vessels. Table 1.1 provides an overview of the factors involved in angiogenesis. Angiogenesis must be balanced to maintain homoeostasis. A number of pathologies are associated with unbalanced angiogenesis (see figure 1.3), either as the primary cause or as a consequence and hallmark of the disease (fig. 1.3).
1. Introduction

1.2. ANGIOGENESIS

Figure 1.3: Pathologies associated with unbalanced angiogenesis.

Table 1.1: Pro- and anti-angiogenic factors. Adapted from Folkman and Kalluri [2004].

<table>
<thead>
<tr>
<th>Anti-angiogenic factors</th>
<th>Pro-angiogenic factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSP-1</td>
<td>VEGF</td>
</tr>
<tr>
<td>Angiostatin</td>
<td>PIGF</td>
</tr>
<tr>
<td>Endostatin</td>
<td>FGF</td>
</tr>
<tr>
<td>Tumstatin</td>
<td>PDGF</td>
</tr>
<tr>
<td>Interferon</td>
<td>EGF</td>
</tr>
<tr>
<td>Integrins</td>
<td>MMP</td>
</tr>
<tr>
<td>TIMP</td>
<td>Low pH and hypoxia</td>
</tr>
<tr>
<td></td>
<td>Angiopoietin</td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
</tr>
</tbody>
</table>

Angiogenesis normally occurs in wound healing and menstrual cycle in re-
spontaneous response to local damage of the pre-existing vasculature. When a vessel lesion occurs one of the first consequences is decreased oxygenation to the affected area; this is known as hypoxia (pO$_2$ between 5 and 10 mmHg, concentration 0.1-3%) and is one of the initiators of angiogenesis (Moeller et al. [2004]). To form new vessels (a) the pre-existing ones are disrupted, (b) the extracellular matrix is degraded, (c) endothelial and perivascular cells migrate in the direction of the new vessels growth and finally (d) the newly formed vasculature is stabilised (Harris [2002]). Vessel destabilisation results from the loss of cell-cell contact between endothelial cells and also with perivascular cells. In response to hypoxia local NOs concentration increases transcription of Hypoxia-inducible Factor-1α (HIF-1α) and this up-regulates Vascular Endothelial Growth Factor (VEGF) production by tumour (Liby et al. [2012]) and perivascular cells (Pinto et al. [2010]). VEGF is a potent pro-angiogenic factor that was first identified by Senger in 1983 and initially called Vascular Permeability Factor (VPF) (Senger et al. [1983]).

VEGF promotes the secretion of angiopoietin 2 from endothelial cells. Angiopoietin 2 is an inhibitor of angiopoietin 1, which stabilises cell-cell contact (Fagiani and Christofori [2013]). Angiopoietin 2 and VEGF, combined with an increased expression of metalloproteases degrading the Extracellular Matrix (ECM), promote vessels instability and increased tissue permeability (Jain [2003]). Once pre-existing vessels are degraded and the extracellular matrix becomes less dense, endothelial cells commence proliferation and migration in the direction of the new vessel sprout. The migration follows a leading endothelial cell called tip cell and is stimulated by VEGF, Fibroblast Growth Factor (FGF) and α$_v$β$_3$ integrins gradients (Cai et al. [2006], Monti et al. [2013], Sato et al. [2012]). Finally the angiogenic process is switched off by the formation of tubular structures (vessels) and cell-cell interactions, particulary endothelial cell-pericyte interactions, promoted by Platelet Derived Growth Factor (PDGF) and angiopoietin 1. Pericytes are recruited by endothelial cells secreting PDGF, angiopoietin 1 and Transforming Growth Factor-β1 (TGF-β1) (Hellstroem et al. [1999]). Vessel stabilisation is regulated by FGF, VE-cadherin, PDGF, TGF-β1 and angiopoietin 1 (Ramsauer and D’Amore [2007], Zhang et al. [2009]). Non functional vessels are disrupted and removed in the final remodelling of the new vasculature (Ichioka et al. [1997]).

### 1.2.3 Tumour angiogenesis

When angiogenesis is not initiated, tumours remain vital but do not exceed $\approx 1mm^3$. When angiogenesis is initiated, tumour growth becomes exponential (Folkman [1971]). Small, dormant in situ tumours form frequently, but do not
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1.2. ANGIOGENESIS

Evolve to invasive cancer. This concept was suggested by Folkman in 1989 and is known as cancer without a disease. It defined angiogenesis as a hallmark for cancer (Folkman et al. [1989]). When the tumour grows, its centre becomes hypoxic. Hypoxia is a major switch of tumour angiogenesis as it promotes the secretion of angiopoietin 2 and VEGF with exactly the same mechanisms described in physiological angiogenesis. It has been observed that both tumours and normal tissues exhibit similar angiogenic mechanisms, with the crucial difference being that tumours inhibit the switch off signal, maintaining the vasculature in an active state of constant remodelling, without final maturation. This allows exponential tumour growth well beyond the limit of $\approx 1 \text{mm}^3$ reported for non vascular tumours. Finally, cancer cells can escape the primary tumour site, enter the blood system and colonise other tissues to establish secondary tumour. This process is known as metastasis and it is associated with a poor prognosis (Riihimaki et al. [2013]).

1.2.4 Tumour vasculature and tumour microenvironment

Tumour vasculature

Tumour vasculature is immature and unstable. There is no well defined arterioles/capillaries/venules hierarchy. Vessels are tortuous and with a heterogeneous diameter and some are blind ended. Finally, since the vasculature is not mature, large fenestrations (up to micrometers) exist between endothelial cells and there is no or minimal pericytes coverage (Diaz-Flores et al. [1992]). As a result of the large fenestrations the tumour vasculature is more leaky than normal vasculature, a key feature in both the primary and metastatic stages of the disease. In addition, the lymphatic drainage is compromised (Matsumura and Maeda [1986]), resulting in increased hydrostatic pressure within the tumour. The combination of leaky vasculature and reduced lymphatic drainage produces Enhanced Permeation and Retention (EPR) and is of great importance in tumour targeting. EPR effect will be discussed in further detail in section 1.4.2.

Tumour microenvironment

The tumour microenvironment consists of the ECM, chemical factors and a variety of cell sub-types. These include fibroblast, endothelial cells, perivascular cells and immune cells which are normal cells but contribute to tumour growth. The region around the tumour is characterised by inflammation, which supports angiogenesis as it has been observed in wounds. This similarity between tumours and wound resulted in the definition of cancer as a wound that does not heal (Dvorak [1986]). Fibroblasts in tumours are more specifically named Cancer Associated Fibroblast (CAF) to distinguish them from normal
fibroblasts. CAF are derived from normal fibroblasts, pericytes, Mesenchymal Stem Cells (MSC), endothelial cells or epithelial cell via a transformation process (Endothelial-Mesenchymal Transition (EndT) and Epithelial-Mesenchymal Transition (EMT) respectively) and are often myofibroblast with increased smooth muscle actin (Fuyuhiro et al. [2011]). CAF actively contribute to angiogenesis by secreting VEGF, PDGF and TGF-β1, all pro-angiogenic factors (see section 1.2.2-1.2.3) (Raesanen and Vaheri [2010], Marsh et al. [2013]). Immune cells such as Tumour Associated Macrophages (TAM) and Tumour Infiltrated Lymphocytes (TIL) are also present in the tumour microenvironment, exhibiting reduced cytotoxic activity compared to their normal counterparts. The reduction of cytotoxic activity appears to be mediated by the cancer cells (Qian and Pollard [2010], Valenti et al. [2007]).

1.2.5 Angiogenesis as a target in cancer therapy

Angiogenesis is a hallmark of cancer. It has been highlighted how cancer growth and survival is dependant on angiogenesis (section 1.2.3). The combination of these two factors makes tumour angiogenesis an attractive target in cancer therapy. There are several anti-angiogenic compounds already approved by the Food and Drugs Administration (FDA) or in clinical trial, including Bevacizumab, Pegaptanib (Vascular Endothelial Growth Factor Receptor (VEGFR)), Cetuximab (Epidermal Growth Factor Receptor (EGFR)). The aim of vascular targeted therapy is to starve tumours by interfering with the disease vascularisation. It has also been proved that traditional chemotherapeutic agents are more effective when combined with anti-vascular agents (Ma and Waxman [2008]). The vasculature can be compromised either by inhibiting the formation of new vessels (anti-angiogenesis therapy) or by disrupting existing vessels (Vascular Disrupting Agent (VDA)-based therapy).

Anti-angiogenesis. The concept of anti-angiogenesis was first introduced by Folkman in 1971 (Folkman [1971]). Perhaps the most studied anti-angiogenic target is VEGF. Initially named as VPF by Senger and co-workers in 1983 (Senger et al. [1983]), and later renamed VEGF by Leung who also attributed angiogenic properties to the molecule (Leung et al. [1989]). VEGF is one of the most potent pro-angiogenic factors. Inhibition of VEGF results in reduced new blood vessel formation and tumour stabilisation or regression. Vessel stabilisation has been reported to improve drug biodistribution to the tumour in a synergistic effect of the antiangiogenesis and chemotherapy combination. Another target for anti-angiogenesis are pericytes, a perivascular cell population controlling vessel
stability. The rationale being that tumours having lower pericyte coverage are more sensitive to drugs targeting the endothelial cells, such as anti-VEGF drugs (Benjamin et al. [1998], Erber et al. [2004]). However this approach has failed in clinical studies because of toxicity (Hainsworth et al. [2007]). An alternative pericyte-targeted approach was proposed by Raza and colleagues, using a pro-pericyte strategy which could be beneficial. By stimulating pericyte recruitment blood vessels will be stabilised and new angiogenic sprouting inhibited. This could also limit metastasis by reducing the leakiness of the vasculature (Raza et al. [2010]).

**VDAs** target the differences between the tumour and normal vasculature, such as increased fragility, proliferation and protein up-regulation. Several VDAs are in clinical trials, including for instance combretastatin (Thorpe [2004]). Combretastatin’s effect is dependent on tumour type and pericyte coverage, suggesting again the importance of pericytes as an additional potential target in vascular therapy. Pericyte-rich tumour models such as implanted Mouse Fibrosarcoma Cells expressing VEGF188 isoform (MFC188) have a greater response to combretastatin compared to tumours with lower pericyte coverage such as Mouse Fibrosarcoma Cells expressing VEGF120 isoform (MFC120) (Tozer et al. [2008]).

Despite it being more than 80 years since Folkman’s anti-angiogenesis hypothesis, more than 25 years since the discovery of VEGF as an anti-angiogenic target and almost 10 years since the approval of the first anti-angiogenic drug there is only a small amount of studies demonstrating clinical efficacy of anti-angiogenesis therapies, even in combination with traditional chemotherapy. Progression free survival is improved but this is not always the case for overall survival. At the time of its approval, overall survival for the VEGF signalling inhibitor Bevacizumab was reported to be increased from 15 to 20 months (Hurwitz et al. [2004]), but later trials failed to reproduce these results (Bellou et al. [2013]). The poor outcome of anti-angiogenic therapy are likely to be due to multiple causes: a) Endothelial cells from different tissues have a different phenotype and therefore are likely to have a differentiated response (Hida et al. [2004]); b) Resistance to VEGF signalling inhibition has been reported (Bellou et al. [2013]); c) The angiogenesis pathway has demonstrated redundancy: the inhibition of one factor or pathway results in the increased secretion of another factor. For instance it has been reported that VEGF inhibition can result in increased Epidermal Growth Factor (EGF) and FGF production (Grothey et al. [2008], Batchelor et al. [2007]); d) Vascular disruption can lead to metastatic tumours (Ebos et al. [2009], Paez-Ribes et al. [2009]). Overall, there is no clear understanding of which patients will benefit from anti-angiogenic therapies due to difficulties assessing the correlation
between drugs and their effect (Ribatti [2010]). Pericytes are undoubtedly involved in the response to anti-angiogenic therapies, however their role is not well understood. As pericytes may protect endothelial cells from anti-VEGF therapy a combination of anti-VEGF and Platelet Derived Growth Factor-B (PDGF-B) therapy have been tested with contrasting outcomes. Some studies supported the validity of a combined approach (Bergers et al. [2003], Erber et al. [2004]), while in other models there was no response, or toxic side-effects were reported (Kuhnert et al. [2008]). In another study using a pericyte-deficient mouse no difference in anti-VEGF therapy response was observed when compared to that of normal mouse, suggesting that the therapy may not be specific to the tyrosine-kinase receptors, but may interfere with other pathways (Nisancioglu et al. [2010]).

1.3 Imaging techniques

Imaging techniques are an essential tool in research as they provide data from direct observation of biological events. In cancer research, imaging has several applications, including a) diagnosis, b) understanding of the mechanism of evolution and spreading and c) evaluation of treatment outcome. Techniques such as X-ray, Positron Emission Tomography (PET) and Magnetic Resonance Imaging (MRI) are used in clinical practice to localise cancer and evaluate size and eventually metastasis. MRI can also be used to obtain functional information relating to blood flow and permeability of the tumour vasculature (Gaustad et al. [2008]). In pre-clinical research, optical imaging techniques based on fluorescence and bioluminescence can be used to investigate phenomena at the cellular and molecular level. The following sections review imaging techniques, their limitations and strategies to overcome these limitations.

1.3.1 Techniques and limitations: an overview

There are a number of different available imaging techniques. Not all of them can be applied to any problem, the appropriate technique should be chosen accordingly to the aim of the study. For example, Transmission Electron Microscopy (TEM) can be used to investigate sub-cellular organelles with nanometre spatial resolution in fixed samples, but cannot visualise the distribution of a radio therapeutic agent in an animal.

The best imaging technique for a specific study should be chosen considering spatial resolution, time resolution, tissue penetration and cost. For a given application, the ideal imaging technique should have enough spatial resolution to visualise the structure of interest, enough tissue penetration to visualise such
structure wherever it may be located within the body, and finally it should have
good time resolution to image events at the time scale desired. Techniques such
as X-ray, MRI, Computed Tomography (CT), PET and ultrasounds are com-
monly used to detect tumours and assess morphological properties such as size
and presence of metastasis (McDonald and Choyke [2003]). Since the original ap-
plication of the techniques, spatial resolution has been greatly improved. Micro-
and nano- bubbles improved spatial resolution of ultrasound from millimetres
to micrometers (Lindner [2004], Yin et al. [2012]). Similar improvements have
been achieved for MRI, by refining imaging acquisition and processing technique
(Brindle [2008]). Thanks to such improvements it is possible to obtain more de-
tailed functional information from MRI. As an example, Brindle and co-workers
were able to detect cell death in a tumour by specifically targeting the delivery of
the contrast agent gadolinium to apoptotic cancer cells (Krishnan et al. [2008]).
Others applications include detection of tumour hypoxia, blood volume and vas-
culature permeability (Pacheco-Torres et al. [2011], Roberts et al. [2000], Cha
et al. [2000], Law et al. [2004]).

Optical imaging such as confocal imaging offers great spatial resolution. In
angiogenesis imaging, wide field analysis allows visualisation and quantification
of morphological parameters such as tortuosity and Micro Vessel Density (MVD),
both important parameters correlated to tumour severity. Improved spatial res-
olution and 3D mapping are possible with Confocal Laser Scanning Microscopy
(CLSM), where the use of a pinhole allows elimination of out of focus light, im-
proving signal to noise ratio. When the focus plane is moved through the specimen
a 3D image can be reconstructed (Pawley [2006]). In Multi Photon Microscopy
(MPM), fluorescence is generated when two or more photons with total energy
equal to the one required by single photon fluorescence excitation are adsorbed
by the fluorophore at the same time. This is an unlikely statistical event to occur,
therefore the beam of light must be highly focused. As a result, only a restricted
plane of focus is generated and no pinhole is required to eliminate out of focus
light (Denk et al. [1990]). The main advantages of MPM over CLSM are greater
tissue penetration (from $\approx 60\mu m$ of CLSM to a few hundreds of micrometers) and
reduced photobleaching and phototoxicity (Lunt et al. [2010]). Despite MPM
being achieved with any number of photons, in practical applications MPM is in
fact dual-photon. However, recently a tri-photon application has been reported
to achieve great spatial resolution and tissue penetration in a mouse brain model
(Horton et al. [2013], Howard et al. [2013]). Spatial resolution can be further
increased using techniques such as Stochastic Optical Reconstruction Microscopy
(STORM) and Stimulated Emission Depletion (STED) (Denk and Webb [1990],
Hell and Wichmann [1994], Rust et al. [2006]) in Intra Vital Microscopy (IVM).
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1.4. Nanocarriers

<table>
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<tr>
<th>Technique</th>
<th>Tissue penetration</th>
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<td>1(\mu m)</td>
<td>Seconds</td>
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<td>Bioluminescence Imaging</td>
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<tr>
<td>FMT</td>
<td>&lt;10(cm)</td>
<td>1(mm)</td>
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</tr>
<tr>
<td>PET</td>
<td>No limit</td>
<td>1(mm)</td>
<td>Minutes</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>cm</td>
<td>50(\mu m)</td>
<td>Seconds</td>
</tr>
<tr>
<td>CT</td>
<td>No limit</td>
<td>50(\mu m)</td>
<td>Minutes</td>
</tr>
<tr>
<td>MRI</td>
<td>No limit</td>
<td>10-100(\mu m)</td>
<td>Minutes</td>
</tr>
</tbody>
</table>

Table 1.2: Imaging techniques comparison by spatial and time resolution and tissue penetration. Data from Weissleder and Pittet [2008].

In traditional optical imaging spatial resolution is estimated with the Abbe equation for the diffraction limit \(D = \lambda/2 \cdot NA\) where \(D\) is the diffraction limit, \(\lambda\) is the wavelength used and \(NA\) is the objective numerical aperture. STORM and STED microscopy bypass the diffraction limit and structures \(10nm\) apart can be resolved. Despite achieving remarkable progress in tissue penetration, optical imaging \textit{in vivo} without further modification can only be applied to study angiogenesis in the retina, where the eye provides a natural transparent window to access the specimen (Wright et al. [2012]).

1.4 Nanocarriers

1.4.1 Background

Cancer targeting for therapy or imaging requires specific extravasation which is controlled by the size of the vessel fenestration. Moreover, especially in therapy, some of the most potent drugs are poorly water soluble and have severe side effects. The combination of the these problems (targeting, solubility, side effects) has lead to the development of nanometre sized carriers.

1.4.2 Targeting tumour with nanocarriers: basic principles

To take full advantage of any imaging technique or cancer therapy based on nanocarriers, it is critical to maximise the delivery of contrast agents or drugs to the tumour site, while minimising distribution to other organs. This operation is known as targeting. In cancer applications, targeting can be divided into two subsequent mechanisms: \textit{passive targeting} and \textit{active targeting}. Passive targeting takes advantage of the irregular anatomy of the tumour micro-vasculature, while
active targeting requires specific nanocarrier/cell recognition within the tumour microenvironment.

Passive targeting

Tumour microvascular anatomy has been described previously (sect. 1.2.4). Tumour blood vessels have fenestrations up to micrometres, while healthy tissue vasculature has no or little fenestration, with the exception of specialised structures such as hepatic sinusoids where fenestration as large as 150\(\mu m\) have been described. Tumour vasculature fenestrations lead to increase vascular leakiness. Moreover lymphatic drainage is compromised. The sum of the two factors is known as the EPR effect and it was first described by Maeda (Matsumura and Maeda [1986]). The EPR effect represented a revolution in tumour therapy and cancer targeting. Delivery agents have been engineered to effectively extravasate into tumours. Using the same mechanisms imaging techniques have been improved too. For instance MRI imaging of tumour is enhanced by contrast agent extravasation and accumulation (i.e. gadolinium) and ultrasound imaging can be enhanced by the accumulation of nanobubbles (Xing et al. [2010]). Unfortunately, increased leakiness and reduced lymphatic drainage cause increased hydrostatic pressure. When intra tumour pressure is higher than intra-capillary pressure, gradient driven diffusion of molecules from the vasculature to the tumour is reduced. In this case diffusion is dependent only on the concentration gradient and the diffusion coefficient, which is inversely correlated to the radius according to the Stokes-Einstein equation \(D = \frac{k_b T}{6 \pi \eta R}\), where \(k_b\) is the Boltzmann’s constant, \(T\) is the temperature, \(\eta\) is the viscosity and \(R\) is the particle radius. Diffusion is a slower process compared to convection. However, since the first description of EPR almost 25 years ago, countless of proofs have been collected in favour of particle accumulation in tumours due to such effect. Delivery systems exploiting the EPR effect include a huge heterogeneity of chemistries, ranging from quantum dots to liposomes, bioconjugates, micelles and polymersomes (Moghimi et al. [2001]). In imaging, passive targeting is useful to reveal the presence of tumour mass. For techniques aiming at higher spatial resolution passive targeting is not enough and specific tumour features should be targeted. This second level of targeting is known as active targeting.
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1.4. NANOCARRIERS

Figure 1.4: *EPR effect.* Tumours blood vessels are characterised by large fenestration that allows nano-particles to extravasate. Furthermore, lymphatic drainage is reduced therefore nano-particles are not rapidly cleared by the interstitial cellular flow. In normal tissues there are no or smaller fenestrations, therefore nano-particles will not extravasate.

**Active Targeting**

By the EPR effect nano-metre sized particles can passively accumulate within tumours. When a therapeutic or imaging agent is delivered specifically to the cell of interest within the tumour the term *active* targeting is used. Active targeting of nanocarriers is achieved by functionalisation with moieties that can recognise specifically a cell type. Active targeting has been extensively researched in therapy (see Moghimi et al. [2001] for an extensive review). Limiting the subject to imaging, as more relevant to the work presented in this thesis, the most remarkable achievements have been reported when active targeting is coupled to high resolution optical imaging such as CLSM or MPM. An extensive review of nanocarriers and different targets can be found at (Brannon-Peppas and Blanchette [2012]).

1.4.3 Nanocarriers pharmacokinetics

The body is highly compartmentalised: organs are confined and separated from each others, normally communicating via the circulatory system. Compartmentalisation is found also at the cellular level, where different organelles are separated from each others by lipidic membranes. Nanocarriers *in vivo* will have to travel through a number of different barriers in order to reach the desired site of accumulation. The number and type of barriers depends on both the administration site and the localisation of the target. The fate of any molecule or carrier
in the body is traditionally studied in terms of the Administration, Distribution, Metabolism, Excretion (ADME) profile.

**Nanocarriers administration** Typical administration routes are *Intra Muscular* (i.m.), *Intra Peritoneal* (i.p.), *Intra Venous* (i.v.) and *Sub Cutaneous* (s.c.). i.m., i.p. or topical administration are useful in local delivery. However, i.v. administration is more common and it is referred to as systemic administration, as the injected substances will be spread to the whole body via the circulatory system. Local delivery is useful to maximise the concentration of the delivered agent at the delivered site, and normally release from cargo is slow and sustained, compared for instance to systemic delivery where nanocarriers are challenged by the *Reticuloendothelial System* (RES) which can quickly clear them from the circulation. The term RES or the equivalent *Mononuclear Phagocyte System* (MPS) refers to phagocytic cells which are part of the immune system. These include macrophages and monocytes, but also hepatic Kupffer cells. Therefore, the RES represents the first barrier that must be overcome to avoid fast blood clearance of nanocarriers.

**Nanocarriers distribution** Nanocarriers delivered systemically will circulate in the blood and distribute to different organs. The profile of distribution is dictated by their size, shape, and charge. Most of the polymeric nanocarriers are cleared by the RES (also referred to as MPS) after opsonisation. Opsonisation is the recognition by a group of plasma protein known as opsonin which includes specific immunoglobulins and complement proteins. Opsonisation is a fast process: it only take seconds. Opsonised nanocarriers are recognised by phagocytic cells and accumulate in liver and spleen, but also lung and kidney. The ideal nanocarrier size for tumour accumulation and MPS is below 100 nm ([Alexis et al. 2008], [Perrault et al. 2009], [Petros and DeSimone 2010]). Nanocarrier shape also affect clearance. It has been reported that tubular micelles exhibit better pharmacokinetic profile than spherical vesicles, probably because elongated shapes are easily up-taken by phagocytic cells ([Geng et al. 2007]).

Chemical composition of the nanocarrier is important because it affects recognition by MPS. For this reason many formulations have a protective brush of *Polyethylene Oxide* (PEO). Another strategy to prolong particle half life is coating with molecules recognised as *self* by the MPS. An example has been reported by Discher and co-workers when they coat microbeads with CD47, a *marker of self* normally found on the surface of red blood cells ([Tsai and Discher 2008]).

At the tumour site, nanocarriers will need to leave the blood stream to enter the tumour and release their cargo. Size is critical in the extravasation step.
Nanocarriers should have smaller size than the pore size cut-off of vascular fenestration in tumour sites, but bigger than fenestrations in normal tissues. Diffusion from the vessels to the tumour is inversely correlated with particle radius (Dreher et al. [2006]). Charge is also a critical factor in diffusion. Cationic particles diffuse faster and further than neutral or anionic counterparts (Dellian et al. [2000]). Finally, using beads functionalised with quantum dots and targeted to HER2, Tada and co-workers were able to visualise accumulation of the beads in the target cells and by tracking their trajectories they extrapolate extravasation kinetic parameters, including an average travelling speed of 1-4 μm/s, characterised by a stop-and-go movement most likely caused by a cage effect of the dense extracellular matrix (Tada et al. [2007]).

At the tumour site, nanocarriers release their cargo either extra-cellularly or intra-cellularly. Extra-cellular release can be achieved by exploiting tumour microenvironment specific features such as low pH (≃6) or high temperature (Kratz et al. [2008]). Cellular up-take mechanisms can occur by phagocytosis only in a few specialised cells. The most common mechanisms of up-take include pinocytosis and endocytosis, mediated or not by receptor-ligand interactions. For an extensive review on nanocarriers endocytosis see Canton and Battaglia [2012]. As biodistribution, cellular uptake is controlled by size, shape, chemical properties and topology of the vesicles, however in some cases ideal biodistribution parameters do not match ideal cellular uptake conditions. For instance, cellular uptake is higher for positively charged nanocarriers because of the interactions with the negatively charged proteoglycans expressed at cellular surface. Unfortunately this is also true for the cells of the MPS, therefore positively charged particles have poor biodistribution. Considering nanocarriers size, it is interesting to notice that the same size that in flow escape the MPS (radius ≤ 100 nm) is also a good size for cellular uptake: smaller particles are up-taken faster in static in vitro endocytosis assays which can resemble to a condition of very low flow at the tumour level. In more detail, cellular uptake requires the cellular membrane to bend to engulf around the nanocarriers. This process requires energy and there is a theoretical minimum radius that particles should possess to facilitate the process: $R_{\text{min}} = \sqrt{\frac{2kA}{\mathcal{E}k_BT}}$ where $k$ is the membrane bending modulus, $A$ is the membrane surface area, $\mathcal{E}$ is the binding energy (e.g. chemical energy per ligand-receptor pair), $k_B$ is the Boltzmann constant, and $T$ is the temperature (Chaudhuri et al. [2011]). Finally, nanocarriers topology affects cellular uptake. In polymeric vesicles it has been found that mixtures of different co-polymers creates domains in the nanocarriers membrane and brush. Such domains affects kinetics uptake (Massignani et al. [2009], LoPresti et al. [2011]).
Nanocarriers metabolism

Metabolism depends on the nanocarrier chemistry and size. For polymeric vectors, biodegradable polymers can be broken down and the monomer can be recycled in the body’s metabolic pathways or eliminated by the kidney or the GI (Mehvar [2000]). Small non biodegradable polymers are secreted by the kidney, provided they are below the glomerular threshold. Glomerular threshold for PEO has been measured to be $30KDa \approx 680$ monomers) with elimination rate inversely correlated to molecular weight (Webster et al. [2007]). If the co-polymer is non biodegradable and below the glomerular threshold it may be secreted with the faeces, however accumulation in the body is a concern and more studies are required to elucidate the fate of polymers, especially concerning chronic administration and renal or liver accumulation.

1.4.4 Nanocarriers self-assembly

Nanocarriers can be made by a number of different chemicals, ranging from lipids to polymers to metals. An important classification can be made on the basis of the type of interaction between the components. Systems such as silica beads, quantum-dots, carbon nanotubes and dendrimers are all characterised by strong covalent bonds between the components. Another class of nanocarriers is characterised by weak interaction between the components based on supramolecular forces, such as the hydrophobic effect, hydrogen bonding, coulomb interaction and aromatic forces. Micelles, liposomes and polymersomes belong to this second class. A key difference between the two classes is represented by the synthetic process. Strong bonds are usually generated via chemical synthesis, while weak interactions mimic self assembly of natural components in water, such as biological membranes, proteins and nucleic acids.

Self-assembly processes involve the aggregation of molecules to form defined structures. As a general rule, this process is concentration dependant, where at low concentrations, the different building blocks are molecularly dissolved and as the concentration increases, they start to interact and self-assemble. This concentration is known as Critical Aggregation Concentration (CAC). Self-assembly can be triggered by several supramolecular interactions that depend on the chemical structure of the components of the carrier and the solvent. Amphiphilic molecules contain a part that is able to interact with water molecules hence is water soluble and a part that are is water repellent and hence is insoluble. The balance between water attracting and water repelling parts will dictate the final structures formed and it is known as packing factor, $p$, defined as

$$p = \frac{v}{a_0 \cdot d}$$
where \( v \) is the volume occupied by the packed hydrophobic block, \( a_0 \) is the surface area per molecule defined at the hydrophobic chain-water interface, and \( d \) is the length of the hydrophobic block (Nagarajan [2002]). Amphiphiles with packing factor smaller than \( \frac{1}{3} \) will generate spheres (micelles), if \( p \) is between \( \frac{1}{3} \) and \( \frac{1}{2} \) cylindrical micelles will be obtained and finally if the packing factor is between \( \frac{1}{2} \) and 1, bilayers (vesicles) will be formed (fig. 1.5) (Bates and Fredrickson [1990], Matsen and Bates [1996b], Matsen and Bates [1996a], Fredrickson and Bates [1996]).

![Diagram of geometrical parameters governing the self-assembly](image)

\[
p = \frac{v}{a_0 \cdot d}
\]

**Figure 1.5:** Geometrical parameters governing the self-assembly. *If the packing factor \( p \) is higher than \( \frac{1}{3} \) the co-polymers self assemble in bi-layer, if \( p \) is between \( \frac{1}{3} \) and \( \frac{1}{2} \) the resulting geometry will be tubular micelles, if \( p \) is lower than \( \frac{1}{3} \) the favoured shape is spherical micelle.*

A common example of *self-assembled* nanocarriers is the liposome. Such spherical vesicles are made by phospholipids. Phospholipids arrange in water in a way that the hydrophobic *tails* are packed together excluding water, while the polar *heads* are at the interface between the water and the hydrophobic tails. The result is a bilayer membrane. Since the membrane cannot extend indefinitely, it will enclose into a spherical vesicle with an hydrophilic lumen.

Liposomes without any further modification, however, have poor plasma *half life* as they are rapidly cleared by the RES. PEO is a synthetic polymer that has been extensively used in bioconjugation to improve *half life* of drugs and proteins *in vivo*. The application of PEO to liposomes technology gave raise to a class of liposomes known as *stealth* liposomes, in which a percentage of the total phospholipids is conjugated with PEO. The construct showed a prolonged *half life* compared to the previous generation of liposomes, however it had stability issues.
The introduction of a highly hydrophilic chain of PEO of molecular weight typically ranging from 2000-5000Da dramatically altered the packing factor toward values \(< \frac{1}{3}\), therefore favouring a micellar shape. For this reason only about 5% of the total lipidic composition was conjugated to PEO, and a 100% conjugation was not possible. To be able to use 100% PEO it is was necessary to replace the phospholipid with an amphiphilic co-polymer (1.6).

![Liposome](image1)

![Polymersome](image2)

Figure 1.6: Critical aggregation concentration (CAC). In Liposomes, free phospholipids and aggregates exists in equilibrium. Functionalisation with PEO increases the value of CAC of the phospholipids, therefore the equilibrium is shifted towards free PEO-phospholipids. As a result the stealth properties are lost over time. Polymersomes, made of 100% PEO-copolymer, have CAC close to 0, therefore their stealth properties are not lost over time.

### 1.4.5 Polymersomes

Polymersomes are nano/micro-metre sized, spherical vesicles characterised by hydrophilic lumen. Polymersomes can be generated by the self assembling of diblock co-polymers, generally referred to as \(A_m-B_n\), where A and B are the hydrophilic and hydrophobic blocks, \(m\) and \(n\) represent the number of monomer in A and B respectively, a critical parameter in the determination of the packing factor. Multi-block co-polymers can also be used to form polymersomes, allowing extra-control on the assembly process, surface and membrane topology (see fig 1.7) (LoPresti et al. [2009]).
1.4.6 Polymersomes applications

Polymersomes can be engineered to deliver hydrophilic moieties encapsulated in the hydrophilic lumen and/or hydrophobic molecules intercalated in the membrane. If a contrast agent such as gadolinium or fluorescent dyes are encapsulated or linked to the co-polymers they can be used in imaging applications, both in vitro and in vivo.

In vitro applications include the intracellular delivery of antibodies and lipids...
for live cell imaging (Massignani et al. [2010]). pH sensitive polymersomes made of Poly(2-methacryloxyethyl-phosphorylcholine)-poly(2-diisopropylamino)ethyl methacrylate) (PMPC-PDPA) were reported to escape endosomes and to successfully deliver functional probes intracellularly (Canton et al. [2013]).

In vivo applications include therapy and imaging practices. In cancer therapy, tumour regression has been reported after i.v. administration of doxorubicin and taxol co-encapsulated in PEO-PLA polymersomes (Ahmed et al. [2006]). Other therapeutic applications include the delivery in vitro of the anti-cancer drug gemcitabine to pancreatic tumour cells via PEO-PCL polymersomes (Sood et al. [2013]) and the use of PMPC-PDPA polymersomes in oral squamous cell carcinoma (Murdoch et al. [2010]).

Figure 1.8: Advantages of the polymersomes. Polymersomes can be loaded with hydrophilic molecules because of the hydrophilic core, they can also incorporate hydrophobic molecules in the membrane, including phospholipids. Finally, they can be functionalise for active targeting or with imaging contrast agent (Christian et al. [2009b], Ahmed et al. [2006], Massignani et al. [2010], Lomas et al. [2010]).
1.4.7 Co-polymers for *in vivo* applications

Blood is a fluid densely packed with cells (e.g. erythrocytes, leukocytes and other circulating cells), nanoparticles (i.e. lipoproteins), proteins (albumin, ferritin), immunoglobulin, small molecules (lipids, sugars, salts). As a result, the free space available for circulating nanocarriers is limited and interactions between them and blood components occur. When circulating proteins interacts and get adsorbed by the nanoparticle the opsonisation process starts, which leads to recognition by phagocytic cells and rapid blood clearance. Alternatively, the nanocarrier might presents superficial features directly recognised by the MPS which increase the rate of particle elimination from blood. In both scenarios, short blood half life of nanoparticles is likely to result in insufficient accumulation in the target organs.

The circulation half life of nanoparticles, specifically polymersomes, can be altered by choosing a hydrophilic co-polymer that prevents polymersome interaction with other blood components. Protection from fast clearance from the blood is a combination of chemical and physical protection. Highly hydrophilic polymers are characterised by a large volume of water molecules acting as a shield. However, charged polymers cause high disturbance of water structure normally given by weaker hydrogen bonds. Moreover, positively charged polymers strongly interact with proteins, which are mostly negatively charged. As a first general rule, the protective hydrophilic polymer should have no charge and should interact with water without disturbing its structure, for example the polymer PEO. PEO is not charged and the oxygen atoms separated by two methylene groups interacts with water via hydrogen bonds. A molecule such as protein or lipid should replace water to interact with the polymer, and this event is energetically not favoured. This chemical protection is however relatively weak and alone is not sufficient to prevent interactions between blood components and the nanocarrier.

In polymersomes, the hydrophilic polymers are packed close to each other, in brush-like conformations and stretch from the hydrophobic membrane towards the exterior (see fig 1.7), generating a physical protective barrier (Vonarbourg et al. [2006]).

In this work, all co-polymers used had the same hydrophobic block (poly(2-(diisopropylamino)ethyl methacrylate) (PDPA)), but different hydrophilic brush: PEO, poly(oligo(ethylene glycol) methacrylate) (POEGMA) and Poly(2 - methacryloxyethyl - phosphorylcholine) (PMPC).

**PEO** is one of the mostly studied *stealth* polymers. It is non charged, highly hydrophilic, and its molecular geometry allows optimal interaction with water via hydrogen binding. This strong hydrophilicity is one of the bases of the *stealth*
properties of the polymer. It was introduced in bioconjugation to prolong circulation half life of molecules in the late 70s.

**POEGMA** is another hydrophilic and non charged polymer. It is chemically related to PEO, however, it is not made by a single long chain of \(-\text{CH}_2\text{CH}_2\text{O}\)-, but of a backbone of poly(methyl ether methacrylate) in which all monomers can be functionalised with short chains of PEO. The resulting polymers is still not fouling (Lee et al. [2009], Stetsyshyn et al. [2013]). *Non fouling* is an important property because it means that blood proteins do not accumulate at the surface of the nanocarrier. Protein accumulation is critical as it leads to recognition by the RES and rapid blood clearance. Another advantage of POEGMA over PEO is that it presents more chemical groups available to functionalisation: PEO only has two ends, one of which is already linked to the hydrophobic membrane, while the other end is usually methoxylated to reduce fouling.

**PMPC** is hydrophilic and zwitterionic: the monomer has a both a positive and a negative charge, therefore it has no total charge, and it can interact with water to form a chemical barrier to opsonisation. Due to its non fouling properties it is widely used in biological applications such as contact lenses and coronary stents. However, it has been found that polymersomes made of PMPC have hydrophilic blocks that are prone to quick internalisation by almost any cell type (Lomas et al. [2010], Massignani et al. [2010], Pegoraro et al. [2014]). Later studies found that PMPC is recognised by scavenger receptors class BI (SR-B1), normally receptors for oxidized low density lipoproteins. This class of receptor is expressed by many cells and it is responsible for the rapid internalisation of PMPC based polymersomes. It should be noted that this mechanism of internalisation occurs when polymersomes directly interact with the cellular receptor SR-B1, and it does not affect its efficacy as non-fouling material in film coatings (Colley et al. [2014]).
1. Introduction

1.4. Nanocarriers

Figure 1.9: The chemical structures of PEO, PMPC and POEGMA. Note how PEO and POEGMA have no charge, while PMPC is zwitterionic. Moreover, POEGMA is characterised by chains of PEO linked to the backbone of the polymer.

1.4.8 Nanocarriers in *in vivo* imaging

Polymersomes have been used in medical imaging due to their capability to accumulate in tumours. In one study Lecommandoux and co-workers exploited the flexibility of polymeric nanocarriers to functionalised PTMC-b-PGA vesicles with a targeting antibody to HER-2. The same vesicles were labelled with a fluorescent tag for *in vitro* cellular uptake study and Ultrasmall Superparamagnetic Iron Oxide nanoparticles (USPIO) were encapsulated in the hydrophobic membrane. This approach allowed them to validate the targeting effect of the functionalisation *in vitro* using Fluorescence Activated Cell Sorting (FACS) and to confirm the *in vivo* accumulation within tumour by MRI (Pourtau et al. [2013]).

In another study Tsourkas and colleagues encapsulated gadolinium conjugated to PAMAM dendrimers into polymersomes of PEO-PBD mixed with the biodegradable PEO-PCL. Polymersomes accumulate into tumour by EPR effect and then partial degradation of polymersomes improves water flux through the membrane. The increased hydration of gadolinium has a positive effect on its relaxation time increasing the signal-to-noise ratio, a common limitation of encapsulated gadolinium. Because gadolinium was conjugated to PAMAM dendrimers its escape from polymersomes and signal dispersion was prevented (Cheng et al. [2009]). PEO-PBD NIR-polymersomes were imaged *in vivo* using optical imaging, allowing
precise localisation and measuring of the tumour size in 3D \textit{in vivo} (Ghoroghchian et al. [2005]). The same formulation was further developed to target dendritic cells \textit{in vivo} by conjugation with the Tat peptide. Dendritic cells were exposed to the polymersomes and then injected in mouse and tracked with an optical imaging set up (eXplore Optix MX2 system) (Christian et al. [2009b]). Another possibility was explored by Battaglia and co-workers by labelling PMPC-PDPA and PEO-PDPA polymersomes with rhodamine, a fluorescent dye that was later tracked \textit{in vivo} with a macroimaging system (Murdoch et al. [2010]).

Polymersomes have also been used in theranostic systems. In this field of research a drug and an imaging reporter are co-encapsulated. For example, PTMC-PGA polymersomes loaded with both the drug doxorubicin and maghemite $\gamma$-Fe$_2$O$_3$ which provides contrast for magnetic resonance. The magnetic properties were also used to drive the polymersomes to the tumour site using an external magnetic field (Sanson et al. [2011]). The anticancer drug paclitaxel and MRI contrast agent super paramagnetic iron nanoparticles have been co-encapsulated into polymersomes to simultaneously deliver the anticancer drug and visualise the tumour response \textit{in vivo} (Muthiah et al. [2013]).

1.5 Aims

Polymersomes are nano-sized carriers successfully developed for \textit{in vitro} and \textit{in vivo} applications, including low resolution optical imaging \textit{in vivo}. However, information on pathological disease at the sub-tissue level requires higher spatial resolution. This can be achieved using confocal microscopy. Because of poor tissue penetration of light, confocal microscopy \textit{in vivo} is not possible at depths greater than a few micrometers. To overcome this limitation, it is possible to use a surgical procedure known as \textbf{Window Chamber (WC)}, in which part of the tissues obstructing the light pathway are replaced with optically transparent glass.

An interesting application of high resolution optical imaging \textit{in vivo} is in tumour microvasculature imaging. Tumour microvasculature development (tumour angiogenesis) is a key step in tumour and metastasis progression. Imaging of the vasculature at high resolution using polymersomes can provide useful insight into the mechanisms governing the diffusion the nanoparticles in the tumour environment, which in turn could be used to increase the fundamental body of knowledge required to design formulations specifically targeted to the tumour microenvironment or specific cell types present.

The aim of this work is to investigate the use of polymersomes in \textit{in vivo} optical imaging coupled with a WC set up. To do so, it will be necessary to
develop a number of screening techniques to test polymersome interactions with cells \textit{in vitro}, and analyse their distribution \textit{in vivo} in the tumour microenvironment. During the \textit{in vivo} screening, efforts will be made to improve the analysis throughput.

Specifically, the \textit{in vitro} development will include quantification of uptake of two different rhodamine labelled polymersome formulations (poly(oligo(ethylene glycol) methacrylate)-poly(2-(diisopropylamino)ethyl methacrylate) (POEGMA-PDPA) and PMPC-PDPA) by three cell types representative of the tumour microenvironment: tumour cells Mouse Fibrosarcoma Cell (MFC), endothelial cells Small Vessel Endothelial Cells (SVECs) and pericytes \textit{in vitro} precursor 10T1/2. The quantification will be conducted both in cells growing in static conditions and in cells growing under flow. Polymersome uptake in flow will be assessed using a specifically developed flow bio-incubator.

\textit{In vivo} applications will focus on the analysis of distribution of polymersomes in the vasculature via image analysis. The analysis aims to highlight the differences between the two polymersome formulations PMPC-PDPA and POEGMA-PDPA in terms of circulation half life and tumour accumulation. These are useful parameters in the rationale design of polymersomes for imaging and therapeutic applications in cancer research.
Figure 1.10: Aim of the work. Different polymersome formulations will be screened in vitro for uptake by different cell lines (10T1/2, MFC188, SVECs. In vitro screening will be performed under flow conditions. Following the in vitro screening, formulations will be tested in vivo using Dorsal Skinfold Chamber (DSC).
CHAPTER 2

METHODS

2.1 Polymersomes

2.1.1 Copolymers synthesis

All co-polymers were synthesized by Prof. Steve Armes’ group, Chemistry Department, University of Sheffield, UK.

PMPC$_{25}$-PDPA$_{70}$ Copolymer synthesis

PMPC-PDPA copolymer was synthesized by Atom Transfer Radical Polymerisation (ATRP) as previously reported (Du et al. [2005]). ATRP is a polymerisation technique characterised by synthesis of copolymers with low polydispersity.

Briefly, 2-methacryloxyethyl-phosphorylcholine (MPC) (1.32 g, 4.46 mmol) and CuBr (25.6 mg, 0.178 mmol) were added with 2 ml of a deoxygenated (30 min with bubbling N$_2$) methanol solution of bpy (55.8 mg, 0.358 mmol) and ME-Br (50.0 mg, 0.178 mmol). The reaction was conducted for 65 min at 20°C in N$_2$ saturated environment. A 3 ml solution of 2-(diisopropylamino)ethyl methacrylate) (DPA) (2.67 g, 12.5 mmol) was injected into the flask and allowed to react for 48 h. The final reaction mixture was diluted with 200 ml isopropanol and purified from the catalyst using a silica column.

Rhodamine-PMPC$_{25}$-PDPA$_{70}$ Copolymer synthesis

Rhodamine labelled (Rhd)-PMPC-PDPA copolymer was synthesized by ATRP as previously reported (Madsen [2009]). Briefly, MPC (1.20 g, 4.05 mmol) was solubilised in 0.75 ml of anhydrous methanol inside a round bottom flask. An in-house prepared rhodamine 6G-based initiator (83.8 mg, 0.135 mmol) was added to the solution and the flask was washed with 0.75 ml anhydrous methanol. bpy
(42.17 mg, 0.171 mmol) and CuBr (19.37 mg, 0.135 mmol) were added to the reaction after degassing with N\textsubscript{2} for 30 min. The reaction was conducted for 40 min at 20°C in N\textsubscript{2} saturated environment. DPA (1.73 g, 8.10 mmol) dissolved in de-oxygenated methanol (2 ml) was injected into the flask. After 48 h, the reaction solution was diluted by addition of methanol (about 70 ml) and opened to the flask. The reaction considered complete when turned green. It was then added of chloroform (200 ml) and purifired form the catalyst using a silica column. bpy was removed by 3 days dialysis against a solution 3:1 chloroform:methanol. The solution was changed daily. After dialysis, the product of the reaction was desiccated, dispersed in water, freeze-dried. Traces of organic solvents were removed by desiccation in a vacuum oven at 80°C for 48 h.

PEO\textsubscript{113}-PDPA\textsubscript{70}

PEO\textsubscript{113} conjugated with the polymerisation macro-initiator Br was dissolved in methanol degassed with N\textsubscript{2} for 30 min. bpy and DPA were added to the solution and the mixture was heated to 50°C before adding the catalyst CuCl. After 16 h an aliquot was analysed by \textsuperscript{1}H-NMR and the reaction was considered concluded when less than 0.1% of monomer was detected. The reaction was stopped by exposing it to air. The reaction mixture was then dissolved in Tetrahydrofuran (THF) and purified from the catalyst using a silica column. The product was then purified from unreacted bpy by dialysis against water for 7 days followed by dialysis against methanol for 2 days. After purification, the product of the reaction was desiccated and characterised by \textsuperscript{1}H-NMR.

2.1.2 Polymersomes synthesis

Polymersomes formulations were prepared by pH switch or film hydration. Both methods can be used to obtain polymersomes, however they differ in preparation time and condition. pH switch is faster, as it can produce milliliters of samples in few hours, while film hydration requires at two weeks of constant stirring. However, because of the faster kinetic pH switch method is characterised by a lower polymersomes yield as micelles formation is favoured. Furthermore, pH switch method requires solubilisation of co-polymers at pH=2, which is not compatible with protein stability and could compromise chemical the binding between co-polymers and functionalising moieties, such as biotin. For this reason the pH switch method was employed in routine preparation of rhodamine labelled formulations, while film hydration was choose in the preparation of biotin labelled polymersomes to prevent hydrolisis of the biotin/co-polymer link. The composition of all the polymersomes formulations used is reported in table 8.9.
Methods

2.1. POLYMERSOMES

Film hydration

Co-polymers were dissolved in chloroform:methanol (2:1 solution) in a sterile glass vial at the concentration of 2mg/ml. The organic solvent was evaporated under vacuum in a desiccator for at least 24h in order to obtain a thin film of co-polymer on the vial wall. The co-polymer film was rehydrated with 2ml of Phosphate Buffer Saline pH 7.4 (PBS\textsubscript{7.4}) and the vial was left under constant magnetic stirring for 2 weeks.

pH switch

20mg of co-polymers were dissolved in a 7ml bijoux with 2ml of Phosphate Buffer Saline (PBS) acidified to pH=2 with Hydrochloric Acid (HCl) 1N. The solution was filtered into a new 7ml plastic bijoux through a 220µm filter. The solution was kept under constant agitation using a magnetic stirrer and a pH meter was used to continuously monitor the pH. 220µm filtered Sodium Hydroxide (NaOH) 0.5N was added to the solution through a programmable pump to ensure slow and constant delivery (18µl/min). The pump was stopped at pH=7.4. All steps were performed at room temperature. Sterile polymersomes were prepared inside a class II cabinet.

Figure 2.1: \textit{PDPA pH sensitivity.} At pH<pKa the tertiary amino group is protonated. The polymer is charged and hydrophilic. At pH>pKa the aminogroups are deprotonated: the polymer becomes hydrophobic.
2.1.3 Polymersomes purification

Polymersomes were purified by Gel Permeation Chromatography (GPC) using 4B Sepharose resin. 500µL of samples were loaded to the head of a 15cm column. Samples were collected in fractions and fractions were characterised by Dynamic Light Scattering (DLS). Fractions containing polymersomes of 100-200nm were joined together. Samples were then concentrated by centrifugation. 1ml was transferred into a 2.0ml eppendorf and centrifuged for 20min at 2000rcf for 20min. The supernatant was transferred to a new 2.0ml eppendorf and centrifuged at 20000rcf for 20min. The supernatant was then discarded and 500µl of sterile PBS was used to re-suspend the pellet.

The suspension was sonicated for 20min to ensure complete re-suspension of the polymersomes. Polymersomes concentration was measured by fluorometer or Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) for functionalised polymersomes and the final concentration was adjusted to 10mg/ml with sterile PBS.

2.1.4 Polymersomes characterisation

Dynamic Light Scattering (DLS)

Size distribution was determined by DLS analysis. Small particles in solution scatters light in all directions. The scattered light at a specific angle can be measured and its fluctuations correlated with time. The intensity of scattered light fluctuates because particles undergo Brownian motion, and smaller particles have higher diffusion coefficients compared to big particles, according to the Stokes-Einstein equation:

\[ D = \frac{k_b T}{6 \pi \eta R} \]

where \( k_b \) is the Boltzmann’s constant, \( T \) is the temperature, \( \eta \) is the viscosity and \( R \) is the radius of the particle.

DLS analysis assumes that particles are spherical. The DLS used was equipped with a He-Ne laser 633nm and was measuring scattered light at an angle of 173°. Briefly, a small aliquot (≈50µl) of polymersome was diluted 1:20 with 220µm filtered PBS, placed into a clean small volume plastic vial and analysed using a Malvern Zetasizer Nano set to 20°C, PBS dispersant and latex beads as particle type. Results are expressed as intensity number against size distribution (fig. 2.2).
2. Methods

2.1. POLYMERSOMES

Figure 2.2: Typical DLS results for PMPC-PDPA and POEGMA-PDPA synthesised by pH switch. Both formulations have average hydrodynamic diameter of \(\approx 150\,\text{nm}\).

Transmission Electron Microscopy (TEM)

Size distribution and morphology of polymersomes were characterised by TEM. TEM is a microscopy technique in which a beam of electron is focused through a thin specimen. In order to achieve optical contrast it is necessary to have electron-dense specimens. In our experiments contrast is achieved with Phosphotungstenic acid (PTA) positive staining. PTA stains the ester bonds present in PDPA and PMPC polymersomes membrane (Gedde [1996]). Polymersomes suspension were diluted to 0.5\text{mg/ml} with 220\,\mu m filtered PBS\textsubscript{7.4}. 400 meshed copper carbon coated grids were charged using a glow charger to allow hydrophilic polymersomes to attach to the grid. 5\mu l of polymersomes were allowed to settle on the grid for 1\text{min} and the excess of liquid was removed using filter paper. 5\mu l of 0.75\%\text{w/v} PTA were added to the grid and allowed to stain for 5\,s. Excess of liquid was removed using filter paper and compressed air. Grids were imaged using a FEI Tecnica Spirit microscope with maximal working voltage of 120\,KV and equipped with Gatan1K MS600CW CCD camera (fig. 2.3).
2. Methods

2.1 POLYMERSOMES

2.1.5 Polymersomes concentration

Fluorometer

Rhodamine labelled polymersomes were prepared including in the formulation 10% w/w of rhodamine labelled copolymer prepared as described in section 2.1.1. The concentration of the rhodamine labelled copolymer was quantified using a fluorometer equipped with a 96 well plate reader. An aliquot (≈20µl) of polymersomes were diluted 20 times with Phosphate Buffer Saline pH 2 (PBS₂). The solution was transferred to a 96 well plate and the concentration of rhodamine labelled copolymer was determined against a calibration curve. Because the polymersomes were prepared starting with 10% molar ratio of rhodamine labelled copolymer the final concentration of polymersomes was calculated by multiplying the fluorometer result by 10.

RP-HPLC

In this work a C18, 150X4.60mm, 5µm, 300Å column was used. The flow ratio was always 1ml/min, the eluents milliQ water and methanol, both acidified with 0.05% of Trifluoroacetic Acid (TFA). The different gradients used are reported in table 2.1 and figure 2.4. An aliquot of 100µl of sample was placed into a RP-HPLC vial and acidified with 2µl of TFA. 80µl of sample were injected and the concentration was determined against a calibration curve.

Figure 2.3: Typical TEM results for PMPC-PDPA and POEGMA-PDPA synthesised by pH switch. Polymersomes are visible as rounded features of ≈150nm of diameter.
2. Methods

2.1. POLYMERSOMES

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Analyte</th>
<th>Wavelength [nm]</th>
<th>Retention time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLY</td>
<td>Rhodamine labelled copolymers</td>
<td>UV 220</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EX/EM 540/565</td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>Rhodamine labelled copolymers</td>
<td>UV 220</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EX/EM 540/565</td>
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<tr>
<td></td>
<td>Antibody</td>
<td>UV 280</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EX/EM 270/300</td>
<td></td>
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</tbody>
</table>

Table 2.1: RP-HPLC settings used.

Figure 2.4: Gradients used in RP-HPLC analysis. a) Gradient POLY, b) gradient AB. Eluent A was milliQ water acidified with 0.05% of TFA, eluent B was methanol acidified with 0.05% of TFA.

2.1.6 Polymersomes functionalisation

Biotin/streptavidin

Biotinylated polymersomes were prepared by film hydration. Composition is available in table 8.9. Polymersomes were then added with streptavidin (1:1 molar ratio with biotinylated copolymer) and kept stirring for 2h at room temperature. Reaction mixture was purified by GPC. The purified reaction was added with biotinylated antibody to Platelet Derived Growth Factor Receptor-β (PDGFR-β) or biotin-TexasRed (1:2 molar ration with streptavidin). The reaction was left stirring for 2h at room temperature. Reaction mixture was purified by GPC and the product was characterised by DLS, TEM and RP-HPLC.
2. Methods

2.1. POLYMERSOMES

Figure 2.5: Biotin Streptavidin functionalisation. Biotinylated polymersomes were added of streptavidin. The excess of streptavidin was removed by GPC. Biotinylated antibody was added. The excess of antibody was removed by GPC.

Maleimide reaction

The reaction was carried out to functionalise polymersomes with Immunoglobulin of the class G (IgG), acting as a model for targeting antibody. A generic rabbit IgG was used instead of a specific antibody to contain costs during the optimisation of the reaction. Composition is available in table 8.9. All liquid reagents were purged with bubbling N₂ for 15 min for every millilitre of solution. Weighted copolymers were dissolved in PBS₂ to a final concentration of 10 mg/ml. pH was slowly increased to 7.4 as previously described (2.1.2), but in this case the reaction mixture was kept under bubbling N₂ to avoid maleimide inactivation by atmospheric oxygen. IgGs were added 1:2 molar ratio with PMPC-PDPA-Mal and the reaction was performed 2 h room temperature. Excess of IgG was removed by GPC. Reaction mixture was purified by GPC and the product was characterised by DLS, TEM and RP-HPLC.

Figure 2.6: Maleimide reaction. Maleimide reacts with thiol groups in IgG.
2.2 Tissue culture

2.2.1 Routine subculture of Murine Fibrosarcoma cells

MFC cells expressing single iso-form of VEGF were kindly provided by Prof. Tozer Group, Oncology Department, University of Sheffield. The development of the cell lines is described elsewhere (Shima et al. [1996], Greco and Dachs [2005]). Briefly, primary fibroblast were isolated from 13.5 days post-coitum Swiss mouse embryo. The cultured fibroblast cultures were genotyped for VEGF-A isoform expression. Finally, retrovirus SV40 was used as vector to immortalise the fibroblasts and oncogenically transform them via H-Ras activation. MFC were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10\% Fetal calf serum (FCS), G-418 600\(\mu g/ml\) and puromycin 2\(\mu g/ml\). Sub-confluent cells were washed with 10\(ml\) PBS, treated with 5\(ml\) trypsin and incubated 5\(min\) 5\% CO\(_2\) at 37\(^\circ\)C. Detached cells were counted using a Neubauer improved hemocytometer and subdivided into cell culture flasks and 10\(ml\) of fresh media was added. Cells were sub-cultured every 3-4 days at a ratio of 1:15

2.2.2 Routine subculture of SVECs

SVECs were maintained in DMEM supplemented with 10\% FCS, 1\% penicillin/streptomycin and 1\% fungizone. Sub-confluent cells were washed with 10\(ml\) PBS, treated with 5\(ml\) trypsin for 5\(min\) and centrifuged. The pellet was resuspended in DMEM and incubated 5\(min\) 5\% CO\(_2\) at 37\(^\circ\)C. Detached cells were counted using a Neubauer improved hemocytometer and subdivided into cell culture flasks and 10\(ml\) of fresh media was added. Cells were sub-cultured every 3-4 days at a ratio of 1:10

2.2.3 Routine subculture of C3H/10T1/2

C3H/10T1/2 were purchased from American Type Culture Collection (ATCC). Cells were maintained in Basal medium eagle (BME) supplemented with 10\% FCS, G-418 600\(\mu g/ml\) and puromycin 2\(\mu g/ml\). Sub-confluent (below 70\% confluency) were washed with 10\(ml\) PBS, treated with 5\(ml\) trypsin and incubated 5\(min\) 5\% CO\(_2\) at 37\(^\circ\)C. Detached cells were counted using a Neubauer improved hemocytometer and subdivided into cell culture flasks and 10\(ml\) of fresh media was added. Cells were sub-cultured every 3-4 days at a ratio of 1:10.
2.2.4 Differentiation of 10T1/2

10T1/2 differentiation into pericytes was stimulated using TGF-β1. In this work the effect of different concentration and time exposure to TGF-β1 was assessed. In every experiment the desired concentration of TGF-β1 was obtained by diluting the stock solution (R&D system) in fresh 10T1/2 media, and then incubating the cells with the TGF-β1 enriched media for the 24h, 48h or 72h at 37°C, 5% CO2. The effect of conditioned media was assessed as well. In these studies 10T1/2 were incubated with media obtained from a flask of MFC188 or SVECs cultured for 72h. TGF-β1 was diluted into the conditioned media before incubation with 10T1/2 cells.

2.2.5 Fibronectin coating

Fibronectin coating of wells was used to assess the effect of different growing substrates in 10T1/2 differentiation and in flow studies to guarantee adhesion of cells under flow. Wells were coated with 5µg/cm² of fibronectin dissolved in PBS7.4. 200µl of fibronectin solution were deposited in a well (24 well plate) and allow to dry at room temperature for 60min. Coated plates were stored at 4°C until use and for no longer than 72h. Wells were rinsed with media before cell seeding.

2.3 Pericyte markers expression quantification

2.3.1 Immunocytochemistry (ICC)

10T1/2, MFC188 or SVECs were seeded at 5000cell/well in dark 96 well plate and incubated 24h at 37°C,5% CO2. TGF-β1 was added to 10T1/2 by replacing the growth media with fresh media added with the desired concentration of TGF-β1. Cells were placed back in the incubator for the desired exposure time.

Conditioned media was obtained from sub-confluent T75 flasks of MFC188 or SVECs cultured for 96h.

At the end of the experiment cells were washed 3 times with PBS7.4 and fixed with 3.6% formaldehyde for 10min. Cells were washed 3 times for 5min with PBS7.4. To assess α-Smooth Muscle Actin (α-SMA) expression cells were permeabilised with 0.1% triton X-100 for 10min and then washed with PBS7.4 for 5min This step facilitate the penetration of the primary and secondary antibody inside the cell, where α-SMA is located. Non specific reactions between primary antibody and cells component other than α-SMA were prevented by incubating cells at room temperature for 1h with blocking solution (5% Bovine serum albu-
mine (BSA) in PBS$_{7.4}$). Primary antibody was dissolved in blocking solution and incubated with cells overnight at 4°C. Cells were washed 3 times for 5min with PBS$_{7.4}$. Secondary antibody was diluted in blocking solution and incubated with cells for 2h at room temperature. Cells were washed 3 times for 5min with PBS$_{7.4}$, DAPI diluted 1:100 in PBS$_{7.4}$ was added to the cells and the plate were stored at 4 °C until imaged. A list of the primary and secondary antibodies used is given in table 8.4. Nerve Glial antigen 2 (NG2) and PDGFR-β were detected using the same protocol, but without the permeabilisation step as they are located on the external cell surface, therefore there is no need to facilitate the penetration of the primary and secondary antibodies inside the cell.

2.3.2 Western blot

For Western Blot (WB) analysis cells were cultured in T75 flasks. Cells were washed carefully with PBS$_{7.4}$, excess of liquid was removed and the proteins were extracted by lysis with 500µl of RIPA buffer for 10min at 4°C. The lysate was transferred to a 2.0ml eppendorf and aggregates were mechanically broken using a syringe equipped with a 30G needle by 10 cycle of aspiration/injection. The lysate was then centrifuged at 4°C for 20min at 10000g and the precipitate was discarded. Protein concentration in the samples was measured using the Bicinchoninic acid assay (BCA) assay, samples were stored in 50µl aliquot at -80°C for further analysis.

**BCA** is a colorimetric assay used to quantify the protein concentration. It is based on a two steps reaction. In the first step cupric acid is added to the sample, and Cu$^{2+}$ is reduced to Cu$^+$ at a rate proportional to the protein concentration. In the second step each newly formed Cu$^+$ ion chelate with with two molecules of bicinchoninic acid. The product of the reaction can be detected by quantifying its optical density at 562nm.

In this work, the BCA assay was used to normalise the loading of all the samples analysed in western blot experiments. Cell lysate were added with the BCA reagents as indicated by the kit supplier for a 96 well plate assay. Samples were incubated 40min at 37°C and optical density was measured at 562nm. Protein concentration was calculated from a calibration curve obtained with a BSA standard.

**Samples preparation** Protein loading was normalised by total protein concentration and loading volume. 20µg of total proteins were loaded in a volume of 40µl. The volume of diluent (RIPA buffer) was calculated as $V_X = 26 - V_S$ were $V_S$ is the volume of stock sample containing 20µg of proteins and $V_X$ is the volume
of RIPA buffer to add. All volumes in µl. The solution obtained was added of 4µl of sample reducing buffer 10X and 10µl of sample buffer 4X. Samples were then denatured at 95°C for 5min and 35µl, and 30µl were loaded into the gel. One channel was reserved for molecular weight ladder.

**Western blot gel preparation** The composition of all gels and buffers is reported in table 8.8. Running gel was poured into a 0.75mm 9 lines template, the template was filled with 2-butanol and the gel was allowed to settle at room temperature for 60min. Excess of 2-butanol was removed and the stacking gel was added on top of the running gel. The system was closed with a template to allow the formation of 9 loading channels. Stacking gel was allowed to settle for 60min at room temperature. The gel was then placed into the running support and loaded into the running tank filled with running buffer.

**Western blot run** Samples were run for 15-20min at 100V to allow uniform sample loading at the top of the running gel. Once samples entered the running gel, voltage was increased to 180V and samples were run until the ladder corresponding to the molecular weight of the protein analysed run for about 2/3 of the high of the gel. This usually took 120min for PDGFR-β and 150min for NG2. When the separation was over, the gel was placed into a semi-dry membrane transfer cassette to transfer the proteins from the gel to a Polyvinylidene fluoride (PVDF) membrane suitable for chemoluminescence staining. Transfer was performed at 100V for 60min.

**Membrane blotting** After transfer the membrane was blocked for 1h at room temperature with blocking solution (5% non-fat milk in milliQ water). The primary antibody diluted 1:500 in blocking solution was left on the membrane over night at 4°C. After washing 5 times for 4min with PBS_{7.4} the secondary antibody diluted 1:10000 in blocking solution was added for 1h at room temperature. The membrane was then washed 5 times for 4min with PBS_{7.4}. The Enhanced chemiluminescence (ECL) solution was added (1ml/membrane) and allow to react for 5min at room temperature. Excess of ECL was removed and the membrane was developed using photographic film.

**Membrane development** Photographic film development was carried out in a dark room. The PVDF membrane was placed into a development cassette. A photographic film was cut to size and placed on top of the PVDF membrane. Exposure time was optimise every time, but it was usually between 30sec and 5min. The photographic film was then placed for a few seconds in developing
solution, washed with tap water and the development was then fixed with fixing solution for $\approx 30 \text{sec}$. The film was washed with tap water and dried at room temperature. The film was scanned and the digital image was used to quantify bands intensity using the *Gel Analyser Tool* available in ImageJ-64bit v1.47. Results are expressed as ratio of band intensity between the sample and the control (10T1/2 cells not treated with TGF-$\beta$1).

### 2.3.3 Quantitative size-based protein assay

Quantitative size-based protein assay was performed using a *Protein simple* equipment commercially known as *Simon*. This machine automates the process of protein separation and quantification aiming to remove user-dependant variables, to make the quantification faster compared to western blot and to reduce costs by reducing the volume of reagents required. This is achieved by a full automation of the process. The user is required to prepare the protein samples and to load them into the machine together with all the other required materials: running and stacking gels, primary and secondary antibodies. When the run starts, the machine automates the process of preparing the gels and to load the samples. Furthermore, reaction with primary and secondary antibody, gel imaging and quantification of protein is also automated by the machine. A schematic of the working principle of *Simon* is given in figure 2.7. Samples are prepared as described for traditional western blot (section 2.3.2), but only 5µl are required for the final analysis. A 384 well plate is prepared by loading a maximum of 12 samples, separation matrix, primary and secondary antibodies and internal standard. The machine automatically loads running capillaries with the reagents and runs the samples according to the specified parameters. In this work samples were run at 300V for 46min, matrix was diluted 80% *v/v* with antibody diluent. All the other parameters are used as default. All reagents are provided by the equipment producer, except RIPA buffer and primary antibodies. Data were normalised with the internal standard and antibody signal peak was integrated using *Simon* dedicated software. Results were expressed as ratio of signal integration between the sample and the control (10T1/2 cells not treated with TGF-$\beta$1).
2. Methods

2.3. PERICYTE MARKERS EXPRESSION QUANTIFICATION

Figure 2.7: Automated western blot work-flow. Samples are loaded into a capillary containing a separation gel. Separation occurs because of proteins different molecular weights and charge as in traditional WB (a voltage is applied to the capillary). At the end of the run the proteins are cross-linked with UV and immunoprobed using a primary antibody and a HRP-labelled sundry antibody. Signal coming from the HRP probe is finally quantified.
2.4 Polymersomes in vitro

2.4.1 Static culture cells treatment

For a typical uptake study, MFC and SVECs cells were seeded on a 24 well plate at a density of 50·10^3 cells density. 10T1/2 cells were seeded at 25·10^3 cell/well and treated for 48h with 2ng/ml of TGF-β1 prior exposure to polymersomes. This different seeding density is required to avoid confluency of 10T1/2, which can inhibit further differentiation and proliferation (Reznikoff et al. [1973b], Reznikoff et al. [1973a]). 500µl of media containing 1mg/ml of polymersomes were then added, cells were incubated at 37°C and 5%CO₂ and uptake was quantified at defined time-points for up to 24h.

2.4.2 Cytotoxicity

Cytotoxicity was assessed by (3 - (4,5 - Dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) assay, a colorimetric method that exploits the ability of living cells to metabolize MTT into a purple formazan salt (see figure 2.8). MFC and SVECs cells were seeded on a 24 well plate at a density of 50·10^3 cells density. 10T1/2 cells were seeded at 25·10^3 cell/well and treated for 48h with 2ng/ml of TGF-β1. 1mg/ml polymersomes dispersed in cell media were incubated with cells at 37°C, 5% CO₂ for 24h cells were washed with 3 times with PBS_7.4. 500µl of MTT 0.5mg/ml were added to each well in a 24 well plate. Cells were incubated 40min at 37°C and 5% CO₂. MTT solution was removed and 300µl of isopropanol acidified with HCl (25µl of concentrated HCl for 20ml of isopropanol). This will solubilise the formazane salts. 150µl of the solution were transferred to a 96 wells plate and the optical density was measured at 540nm (referenced at 630nm). The optical density measure correlates linearly with the cell viability and results are expressed as percentage of viability with respect of a control (cells treated with PBS_7.4, the polymersomes diluent). In in flow studies the 100% viability control was defined as the viability of the same cell line cultured in static conditions.
2.4. POLYMERSOMES IN VITRO

2.4.3 FACS

Cells were detached using a Tripsin/EDTA (TE) cell scraper, transferred to a eppendorf tube and centrifuged at 1000rcf for 5min. From now on every step was performed keeping the cells at 4°C. The pellet was washed with 1ml of ice cold 220μm filtered PBS and cells were centrifuged again. This step was repeated 3 times. The pellet was then re-suspended with 200μl of ice cold PBS and analysed using a BD FACSArray equipment. Cells were gated for size and granularity and for fluorescence intensity using untreated cells as a negative control.
2.4.4 RP-HPLC

RP-HPLC was used to quantify the amount of polymersomes internalised by cells. Because the polymersomes used were 10% rhodamine labelled it is possible to use this chromatography technique coupled with a fluorescent detector to accurately quantify the amount of copolymer present in the sample. To use this technique, it is essential to extract the internalised polymeric material from the cells and solubilise it in a solvent compatible with the RP-HPLC equipment used. This section presents the method optimised to extract the co-polymer from the cells and to quantify it by RP-HPLC. The RP-HPLC gradient used is reported in figure 2.11. After polymersomes treatment cells were washed 3 times with PBS$_7.4$ and excess of liquid was carefully removed. Cells were then left at -20°C for 24h. This step cause cell lysis because of the formation of water ice crystals. 200µl of PBS$_2$ were added to the well and using a 200µl tip as a scraper cells were mechanically broken and the suspension was transferred to a 2.0ml eppendorf. The liquid was centrifuged for 30s at 20000rpm. 150µl of supernatant were transferred into a RP-HPLC vial and 80µl were injected for quantification. RP-HPLC settings are reported in figure 2.11. The workflow of the method is illustrated in figure 2.10. The recovery efficiency, critical to accurate quantification, was assessed to be > 95% with respect of maximal theoretical recovery when a known amount was incubated for 24h at 37°C with cell lysate (see fig. 2.12).

![Diagram](image)

Figure 2.10: Work-flow of polymersomes extraction from cells for RP-HPLC analysis. Cells are lysate by incubation at -20°C for 48h. The co-polymer is recovered by solubilisation in PBS$_2$. 

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2.4. POLYMERSOMES IN VITRO

Figure 2.11: *RP-HPLC* gradient and chromatogram for rhodamine labelled polymersomes. The insert represents the gradient. Free rhodamine and rhodamine labelled co-polymer were resolved in sharp peaks.

Figure 2.12: Recovery of RhdPMPC-PDPA from cells. Rhd-PMPC-PDPA was efficiently recovered (yield >95%) from cells incubated with the co-polymer (treatment) compared to the theoretical 100% total recovery derived from the calibration curve.
2.5 In flow uptake studies

2.5.1 Flow modelling

The flow chamber was developed based on the geometry of a 24 well plate. The flow was delivered using a peristaltic pump Watson Marlow 205S connected to the well with a marprene tube, 0.8 mm bore. The rotation speed of the pump can be tuned to obtain different flow rates. The rotation speed and flow rate were calibrated by collecting different volumes of liquid from the outlet at different time points. The flow inside the chamber was simulated using a finite element approach and COMSOL Multiphysics, Computational fluid dynamic (CFD) module. Different design parameters such as depth of inlet and outlet and the use of a shield to protect cells from direct perpendicular flow were tested in silico using such approach. General simulation settings are reported in table 2.2.

<table>
<thead>
<tr>
<th>General boundary condition</th>
<th>No slip, $u = 0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density $\rho$</td>
<td>$1000kg m^{-3}$</td>
</tr>
<tr>
<td>Pressure $p$</td>
<td>$101325Pa$</td>
</tr>
<tr>
<td>Temperature $T$</td>
<td>$310.15K$</td>
</tr>
<tr>
<td>Inlet conditions</td>
<td>$L_{ent} = 0m$</td>
</tr>
<tr>
<td></td>
<td>$L_{ent}\nabla_t \cdot [-p_{ent} I + u(\nabla_t u + (\nabla_t u)^T)], \nabla_t \cdot u = 0$</td>
</tr>
<tr>
<td>Laminar flow</td>
<td></td>
</tr>
<tr>
<td>Outlet conditions</td>
<td>$L_{exit} = 0m$</td>
</tr>
<tr>
<td></td>
<td>$L_{exit}\nabla_t \cdot [-p_{exit} I + u(\nabla_t u + (\nabla_t u)^T)], \nabla_t \cdot u = 0$</td>
</tr>
<tr>
<td>Laminar flow</td>
<td></td>
</tr>
<tr>
<td>Initial flow</td>
<td>Flow inlet=Flow outlet, $2 \cdot 10^{-8} m^3 s^{-1}$</td>
</tr>
<tr>
<td>Fluid</td>
<td>Newtonian fluid, non compressible</td>
</tr>
</tbody>
</table>

Table 2.2: In silico parameters.

COMSOL shear rate $\phi$ output was converted to shear stress $\tau$ assuming the media as a newtonian fluid: $\tau = \mu \cdot \phi$. The shear stress and velocity magnitude were analysed at the level of cellular growth (bottom of the well).

2.5.2 Device sterilisation and preparation

The well plate was replaced with a new one for every experiment. The outer part of the tubing and the lid were routinely sterilised using azo-wipe. The inside of the tubing and the lid were sterilised by submersion in 70% Industrial Methylated Spirit (IMS) over-night, followed by 2h of slow flow when submerged in
trigene. Finally, the trigene was substituted with 70% IMS for 1h and eventually with sterile PBS for 1h. The PBS was changed every 15min. At this point the device, already kept inside a class II cabinet, was ready to be used. Prior inserting the tubing in the wells the system was equilibrated with cell media for 30min.

2.5.3 Cell seeding

MFC188 and SVECs were seeded in 24 well plate at a density of 50-10^3 cell/well 24h before incubation in flow. 10T1/2 were seeded at a density of 35-10^3 cell/well and 24h after media was replaced with fresh media added with 2ng/ml of TGF-β1 and incubated for 48h before applying flow. Flow was increased to the analysis flow rate at a rate of increased of ≃3.5µl/s every 60min. The two cell lines were seeded at different densities because 10T1/2 are sensitive to confluency, which should be avoided. Therefore they were seeded at a lower density compared to SVECs.

2.6 Polymersomes in vivo

2.6.1 DSC

Animals

Experiments were performed on male SCID mice weighing 30±3g obtained from the University of Sheffield Field Laboratories and held in the animal facility for at least 1 week before experimentation. Prior to surgery, animals were housed in pairs on sawdust, in Perspex cages in a holding facility maintained at 21.5±1.5°C, 40-65% relative humidity, with a 12/12 hour light/dark cycle. Food in the form of standard pelletted commercial diet and tap water were available ad libitum. All procedures were performed under the Animal Scientific Procedures Act (1986).

Spheroids preparation A tumour was implanted in the window of the dorsal skinfold chamber in the form of a spheroid obtained from MFC188. Sub-confluent cells were detached from a flask and counted using a Neubauer counting chamber. Cells were re-suspended to a concentration of 150-10^3 cells/20µl and 20µl drops were deposited on the lid of a 3cm Petri dish. Cells were then incubated 48h at 37°C 5% CO2 to allow spheroids to from in the drops. Humidity was assured by putting 2ml of media at the bottom of the Petri dish.
DSC Surgery

Immediately prior to surgery the warming pad and the site of the surgical preparation were sprayed with Trigene. The chambers, nuts, screws and spacers were sterilised by autoclave. The window chamber was custom made by the University Workshop, University of Sheffield. The parts of the chambers are pictured in figure 2.14. The frame is made of titanium and weights \(\approx 2g\).
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2.6. POLYMERSOMES IN VIVO

Figure 2.14: Parts of the custom made window chamber. The two black frames holding the flap of skin are visible, together with the bolts and the clip holding the optical glass. Bar 7mm.

**Anaesthesia** Animals were anaesthetised with an i.p. injection of a mixture of Hyponorm, 1 part of Hypnovel and 2 parts of injectable water. 100µl per 30g animal were used.

**Shaving** All the steps of the surgery were performed with the animal lying on a hotplate heated at 37°C. The animals dorsal area was shaved from hip to shoulder and around the edge of the ventral area using shaving clippers. Remaining fur was removed with Veet shaving cream applied for 5min and removed with dH2O and gauze swabs. Skin was sterilised with hibi-scrub and alcohol gauze pad. The area where the chamber sits was marked using a sterile marker pen.

**Placement of the chamber** A flap of skin was sutured with 4/0 Ethilon suture to a C-clamp to keep the skin evenly tensioned before applying the chamber. 6 evenly spaced suture points were used. Using a template, the position of the 3 screws and the glass window was marked with a sterile marker pen. A 0.5cm circle of tissue (epidermis, dermis, subcutis, muscle and subcutaneous fat) corresponding to the area of the glass window was removed, leaving blood vessels exposed. Tissue drying was prevented using saline. Finally, the two frames of the chamber were sutured on the two sides of the flap of skin using ethibond suture. Tumours were implanted by placing one spheroid in the middle of the
exposed vasculature of the window. A glass coverslip was placed in the chamber and secured with a spring grip.

**Post operative care and husbandry**  Aureomycin ointment was applied to the chambers frames edges, 100µl of Vetergesic (buprenorphine 30µg/ml) were administered i.p. and 1ml of dextrose/saline was given subcutaneous to aid recovery. Animals were kept in individual cages in a 30°C cabinet. Animals were monitored daily for signs of infections or distress.

**Intra vital microscopy (IVM)**  Imaging sessions started when tumour grow to cover around 80% of the chamber and developed a visible vasculature, typically 7 days post surgery. 80µl of 10mg/ml rhodamine labelled polymerosmes were injected i.v. into the tail vein. Animals were restrained but not anaesthetised during imaging sessions. One imaging session lasted ≃15min. Imaging sessions were performed routinely for up to 24h after injection. Images were acquired using a Zaiss confocal microscope equipped with a 10X objective and a Nikon epi-fluorescence microscope equipped with a 2.5X and a 20X objective.

### 2.7 Statistical analysis

All *in vitro* experiments were performed at least in triplicate (N=3). Statistical analysis and comparisons of groups were performed in MATLAB using the *Statistical Analysis Toolbox*. Experimental error is expressed as standard deviation. Statistical comparisons between groups was performed with a Mann-Whitney *U* test. This non-parametric test is a strong test for difference in mean between two independent set of data non-normally distributed. For normally distributed data, it is comparable to the parametric Student *t*-test. Therefore, as data were not assessed for normality, the Mann-Whitney *U*-test was preferred to the Student *t*-test. Significance level was set to *p*<0.05.

*In vivo* experiments were performed in *N*=1 for PMPC-PDPA imaged at the confocal, *N*=2 for POEGMA-PDPA imaged at the confocal and *N*=2 for POEGMA-PDPA imaged with the epifluorescence microscope. Error was calculated as error = \[\frac{\text{animal}_1 - \text{animal}_2}{2}\] when *N*=2. Because of the small sample size, animal experiments should be considered as pilot experiments, and no statistical significant conclusion can be inferred from the results.
Abstract

Quantification of polymersomes uptake in vitro and intra-tumour distribution in vivo relies on dedicated fluorescence image analysis tools capable of high throughput. Such methods are based on image segmentation to identify Regions of interest (ROI)s such as cell cytoplasm or nucleus, regions of a well corresponding to flow parameter, blood vessel or tumour stroma. The spatial information relative to a specific ROI must finally be correlated with the relative fluorescence signal intensity. The following chapter will focus on the development of such analytical tools.

3.1 Introduction

The large amount of images to analyse requires automation of the method to make the analysis faster and more reliable. Of the many possible alternatives in image analysis, such as ImageJ, R or other commercially available software, MATLAB was chosen as the developing platform. This because MATLAB has a strong Image Analysis toolbox and it is highly customisable through the integration of a number of scripts and functions.

All the software developed are based on segmentation: the extraction of particular ROI from the rest of the image. The segmentation process can be implemented with a number of algorithms, all of which are targeted for enhancing the contrast between the ROI and the background. The algorithms applied and the order they are applied in is highly dependent on the original image. A good confocal image can be segmented using a simple threshold approach: only regions with pixel intensity higher than the threshold are considered. Other images,
such as vasculature images here analysed, do not have enough contrast to use a thresholding masking. In this case, other filters should be applied to facilitate segmentation. Such filters usually operate in multi-step, first blurring the image to eliminate noise, then enhancing the contrast at the edges of the ROI and finally restoring the original sharpness.

In this work, several different pictures (Immunocytochemistry (ICC), up-take, vasculature in vivo) have been acquired using different microscope set-ups (epi-fluorescence, confocal, different magnification). As a result, the analysis tool should perform the same task (segmentation and intensity-based analysis) using a flexible approach, to allow satisfactory results in different conditions. This result was obtained by implementing different segmentation tools into a software with Graphic user interface (GUI) that allows flexible interaction of the user with the masking algorithms.

3.2 Image segmentation

3.2.1 Thresholding masking

The thresholding masking is the simplest approach in segmentation problems. In good confocal or epi-fluorescence images, the background has signal intensity close to 0, while the feature of interest is characterised by brighter regions. When a thresholding masking is applied, the ROI is identified by all the areas having intensity value higher than the threshold. In the software GUI, this was implemented by a slide bar that the user can move to adjust the threshold. A limitation of this approach is that the background should be highly homogeneous and there should be enough contrast between background and ROI, otherwise it is impossible to discriminate the two area. In the analysis here performed, this was achieved only in nuclear DAPI staining, where nucleus are homogeneously stained and the contrast DAPI/background is relatively high (see fig. 3.1). Images characterised by weaker signal and lower contrast, such as low polymersomes uptake or blood vessels, were segmented using different algorithms.
3.2.2 Difference of gaussians (DoG)

DoG algorithm is a form of band-pass filter (Marr and Hildreth [1980]) commonly used to detect edges. Its operating principle is that Gaussian blurring of an image only excludes high frequency spatial information: noise. By applying to the original image two different Gaussian blurring with different standard deviation and subtracting the two blurred images from each other, it is possible to remove noise, and identifying the area of the images corresponding to the lower difference of Gaussian (minimum of the function) allows to accurately identify the edges of the ROI. In MATLAB, a Gaussian convolution kernel is generated with

\[
gaussian = \text{fspecial ('Gaussian', hsiz, sig)};
\]

Where \( hsiz \) define lowpass filter size, and \( sig \) define the standard deviation of the Gaussian. The difference between two kernel is obtained using

\[
dog = \text{gaussian1} - \text{gaussian2};
\]

Finally, the original image is convoluted with the DoG filter using

\[
d = \text{conv2 (double (I), dog, 'same');}
\]

The minimum in the resulting \( d \) matrix represents the regions of the image to be analysed. The DoG algorithm is implemented in MATLAB using two slide bars that allow the user to define the standard deviation of each of the two Gaussian, and a third slide bar to define the \( hsiz \) parameter, common between the two gaussians. Note that the DoG is not an absolute value: inverting the values of \( sig1 \) and \( sig2 \) generates the negative of the mask, i.e. identifies the background rather than the ROI. Figure 3.2 report an example of the application
of the DoG algorithm. Note that the image refers to a micrograph acquired with a 20X objective, which was the most difficult to segment because of the poor background. However, the segmentation analysis did allow to correctly identify the blood vessels. A limited amount of wrongly assigned pixels can be seen around the micrograph edges, but those areas can be excluded by the analysis applying one the filters provided with the developed software.

Figure 3.2: Example of DoG application on a vessel image acquired in bright field using a 20X objective. The original image on the left has poor contrast and cannot be segmented by simple intensity thresholding. DoG algorithm was capable of correctly identifying vessels (right image, in white). Bar 70µm.

3.2.3 Local threshold entropy (LTE)

The LTE algorithm was used to segment some of the in vivo images. It was proposed by Fan and co-workers (Thitiporn and Guoliang [2003]), and it was here slightly modified to meet the specific requirements of the analysis. LTE is a two steps algorithm. The first step is a matched filtering, followed by local entropy thresholding. Matched filtering is an enhance filter that enhances contrast of blood vessels by detecting linear segments in the image (vessels) and assuming Gaussian distribution of intensity at the edges. Once the contrast has been enhanced at the edges of the ROI using the matching filtering, the LTE automatically detects the optimal thresholding for vessel extraction. This is done locally on small regions of the image by assuming that the intensity values are spatially correlated and not pixel-position independent: the ROI is a contiguous region of space characterised by a local homogeneous distribution of intensity values. This is different to the intensity thresholding approach as two different images with the same overall signal intensity but different spatial pixel distribution will have different threshold. A critical parameter is the size of the matching filter, which is correlated to the size of blood vessels. In early versions of the segmentation
software the user was given the possibility to tweak the parameter, however it was later found that results were not better or faster than DoG filtering. The only exception was in segmentation of vessels images acquired with the 2.5X objective. Those images were effectively masked by the algorithm proposed by Fan using the same size of the match filter, most likely because of the resemblance with the images analysed in the cited work, at least regarding blood vessels pixels size.

![Original image](image1.png) ![Mask](image2.png)

Figure 3.3: Example of LTE. An image of blood vessels acquired with a 2.5X magnification was effectively segmented using the LTE algorithm. Extracted vasculature on the right, in white. Bar 800µm.

### 3.2.4 Mask post-processing

**Post-processing intensity thresholding** Regardless of the segmentation process used, the mask generated was not perfect. It could contain some noise given by isolated bright pixels or in some pixels the signal intensity was out of scale (saturated pixels, intensity=255 in an 8bit image). All those values can negatively interfere therefore they should be removed. Saturated pixels were removed by applying a thresholding filter on the mask, and the threshold level was set by the user as previously described. There was also the possibility to remove very dark pixels, an option useful in the clean up process of in vivo images, where some dark pixels occasionally generated unwanted ROI.

**Size-filtering post-processing** Isolated pixels or small groups of isolated pixels (noise) can be removed by applying a size filter to exclude regions smaller (or
bigger) than a defined size limit. This feature is based on the internal MATLAB function `regionprops` and its optional argument `Size`. The `regionprops` command accepts as input a binary image (mask), where white represents the ROI and black the background. The function identifies separate ROIs defined as continuous, uninterrupted regions of white, and store their size values separately. The size filter then check for the size values and excludes ROI outside the size boundaries. T size filter is implemented in the GUI with the use of two side bars that allow to interactively fix the size threshold (upper and lower limit).

![Original image](image1.jpg) ![Cleaning mask, 1st step](image2.jpg) ![Cleaning mask, 2nd step](image3.jpg) ![Final mask](image4.jpg)

Figure 3.4: Original picture (a) was segmented. The mask obtained (b) was cleaned by eliminating high intensity values (c) and small, isolated pixels (d). Bar 22µm.

### 3.3 Pericyte markers image analysis

Images were segmented using the specifically developed software. The workflow of the analysis and a segmentation example is reported in figure (3.5). Nuclear mask was subtract from the cell mask to exclude any cross-talk between the channel by excluding the nuclear ROI from the cell mask. To be able to compare different sets of experiments, the ratio of the average intensity in samples over
the control was used rather than the absolute intensity value. An example of such operation for 10T1/2 treated with 0, 2 or 5 ng/ml of Transforming Growth Factor-β1 (TGF-β1) is reported in figure 3.5-3.6.

Figure 3.5: Work-flow of image analysis for ICC experiments. Cells were segmented using the antibody channel (A, C) to identify the cells and the DAPI channel (B, D) to identify the nucleus. The nucleus masks (F, H) were subtracted to the respective cell masks (E, G) and the average intensity was measured in the resulting ROI (I, J). Bar 22µm.
3.4 Image analysis for uptake in static conditions

Uptake quantification was performed by quantifying the average intensity of cells detected by the segmentation software and plotting the result as a variation of the average intensity over time. To be able to easily compare results between different cell lines, data were expressed with respect of the maximal uptake, fixed as a 100%. The maximal uptake is here defined as the maximal value of intensity recorded for the analysis, which correspond to the uptake of rhodamine labelled Poly(2-methacryloyloxyethyl-phosphorylcholine)-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA) polymersomes from the tumour cell type Mouse Fibrosarcoma Cells expressing VEGF188 isoform (MFC188) at t=24h. An example of this application using modified pictures used in the testing phase is reported in figure 3.7.
3.5 Image analysis for uptake in flow conditions

This analysis aimed to correlate physical flow parameters in specific area of cell in the imaged plate with polymersomes uptake. Images of different areas of a 24 well plate were imaged and the measured uptake was correlated to shear stress or velocity magnitude in the same area.

3.5.1 BD Pathway 855 microscope set-up

The analysis depends on accurately mapping the imaging area of the 24 well plate. Because the BD Pathway 855 does not natively support 24 well plate the well was manually mapped and the imaging positions were saved into the microscope settings as xy offsets from defined positions. The mapping was performed
by accurately drawing a grid on the bottom of the well and using the intersection of the grid as guideline for setting the offset. The grid also allowed us to accurately identify the position of the inlet and the outlet (see fig 3.8).

Imaging a full 24 well plate and all the well used in one analysis required a long amount of time, which was not compatible with cell viability in the microscope. Cell fixation also proved to not be an option, as the fixation severely compromised rhodamine signal (see fig. 3.9) To overcome this limitation, a lower resolution objective was used (10X) three representative areas were imaged instead of the whole well.

![Reference grid](image1)

![Well mapping](image2)

![Overlay with shear stress](image3)

**Figure 3.8: Mapping a well.** Using a well with marked position as a reference (a) images were taken and then a well was reconstructed saving on the microscope software the xy offset (b). Finally, the shear stress mapping obtained from Comsol MultiPhysics simulation was overlaid (c). Bar 3mm.
3.5.2 **In silico** well mapping segmentation

*In silico* simulations was used to quantify shear stress and velocity magnitude at the cell level. Because COMSOL is based on finite element analysis, it divides the volume to be analysed (the well) into a finite mesh, and when asked for the value of shear stress or velocity magnitude at the cell level it only gives the values at the intercept between the mesh and the plane of cell growth. Depending on the size of the meshing this can be a rather sparse matrix. To obtain a detail reconstruction of the well mapping the data were passed to MATLAB and the function `meshgrid` was called to interpolate missing data to the tenth of micrometer resolution, significantly higher than the resolution achievable with the 10X objective used in later imaging. The resulting map was stored as grey scale image and used for segmentation. The range 0-255 was then divided into 8 ROI each one of step 32, so that the first area had intensity 0-31, the second 32-63 etc. Because it is a grey scale 8bit image, the intensity values will range from 0 to 255, corresponding to low and high values of shear stress and velocity magnitude. Knowing the corresponding values it is possible to directly correlate the intensity value to the actual shear stress or velocity magnitude. For instance, if the range 0-255 corresponds to a range of velocity 0-80µm/s, then it can be derived that intensity value of 32 will correspond to velocity \( \frac{80 \cdot 32}{255} \approx 10 \mu m/s \) (fig. 3.10)
3.5.3 Image analysis

After image acquisition, the images were carefully overlayed with the well mapping of a defined flow parameter (shear stress or velocity magnitude). Average signal intensity of cells growing in area with a specific flow parameter was finally correlated with the flow parameter itself, to identify a correlation between up-take and shear stress or velocity magnitude (see fig 3.11).
Figure 3.11: Image analysis method. The first four of the total eight ROIs corresponding to the lower values of shear stress are highlighted in the figure (top right), from dark blue to pale green. A micrograph is placed on the appropriate xy position of an entire well and the 8 ROIs are overlaid to the micrograph (the red lines represents the edges of the single ROI. Intensities of the areas belonging to a ROI are averaged and a new well-uptake image is reconstructed based on the average value).
3.6 Image Analysis for \textit{in vivo} experiments

3.6.1 Masking

\textit{In vivo} image analysis had the aim to quantify and localise signal intensity of rhodamine labelled images of tumour vasculature. This is a segmentation problem as it requires identification of at least two ROIs in every image: vasculature and stroma. The identification of these two ROIs in confocal pictures is not an easy task, mostly because of the very variable and weak signal, especially at later time points. However, the confocal microscope used can acquire simultaneously a transmitted light image of the vasculature, where the vessels are always clearly visible in black over brighter tumour stroma. The simultaneous acquisition together with imaging in different areas of the tumour over-time eliminates the need for registration. Tumour images were segmented using a software specifically developed. First of all, the tumour area to analyse was manually chosen (this option included the possibility to analyse the whole as well), finally intensity threshold, LTE and DoG algorithms were implemented to allow vessel segmentation. Different images were segmented better with different algorithms, depending on the image characteristics (see fig 3.12).
Figure 3.12: Mask generation in different pictures. Images of vasculature acquired using transmitted light in an epi-fluorescence microscope (20X (a), 2.5X (c)), and in a confocal (10X (e)). Images (a) and (b) were both segmented using DoG algorithm. Image (c) was segmented using LTE algorithm. For all images, vessels were correctly identified. Bar (a) 70µm; (c) 800µm; (e) 200µm.

As in ICC and uptake image analysis, masks were further processed to reduce
noise by size filtering. A crucial difference between the ICC and uptake image analysis software and the in vivo image analysis software is the introduction of a third ROI in the latter. This third ROI correspond to the perivascular region and it is therefore located between the vessel mask and the stroma mask. There are two main reasons to justify the introduction of this third mask. First of all, in low resolution images such as 2.5X images, it can be used as a safe zone, to exclude pixels which cannot be assigned to the vessel or the stroma mask because of the low resolution. The second reason is that, in higher resolution images, it can be used to assess the distribution of polymersomes in perivascular region, where pericytes are normally located, and also to evaluate the diffusion of polymersomes into the tumour by measuring the average intensity in this region, which can be dynamically expanded (fig. 3.13).
3. Image analysis 3.6. IMAGE ANALYSIS FOR IN VIVO EXPERIMENTS

(a) Original image, transmitted light  (b) Original image, fluorescence

(c) Transmitted light and mask  (d) Fluorescence and mask

Figure 3.13: Masking blood vessels. Original images on top (a-b), masks overlay at the bottom (c-d). Masks were created on transmitted light images (a-c) and then transferred to rhodamine images (b-d). The tumour area was identified manually and is shown as coloured RGB overlay. Vessels were identified automatically by the proposed segmentation algorithm. Vessels in red, stroma in blue and perivascular region in green. Bar 210 µm.

3.6.2 Vessel mask segmentation: Isolating vessels to determine vascular diameter and diffusion profile

To correlate diffusion of nanoparticles with vessel diameter and travelled distance it is necessary to separate all the vessels identified with the vascular mask. This was achieved by reducing the vasculature mask to its skeleton using the MATLAB built-in function `bwmorph`

```
skeleton_ves = bwmorph(ves, 'skel', inf);
```
The skeleton mask is a binary mask where the vessel has value 1, the non-vessel has value 0. Considering the vessel a linear sequence of 1s, vessels ends and branching points can be easily identified as the only one surrounded by 1 or 3 1s respectively (see fig 3.15). By subtracting the branching points from the skeleton mask a new mask will be created where the continuity of 1s sequence is interrupted at the branching level. The `regionprops` function can be called at this point as it will identify every different ROI, where ROI is defined as a continuous region of space completely surrounded by 0s.

```matlab
list_vessels=regionprops(skeleton_ves_new,...
    'PixelIdxList','Area','PixelList');
```

Note that since it is a skeleton mask running from vascular node to vascular node, the `Area` corresponds to the vessel length. Finally, the function `bwdist` can be called on the negative of the vessels mask to calculate the Euclidean distance between every pixel of the vessel and the closest pixel belonging to the tumour stroma:

```matlab
edtImage = 2 * bwdist(~ves);
```

Note that the multiplicative factor of 2 will directly give the diameter of the vessel. For every vessel, the diameter calculated in the middle of the vessel was stored.

Finally, the Euclidean distance of every pixel of the tumour stroma from the closest vessel is calculated in a similar way:

```matlab
[distances, label] = 2 * bwdist(~stroma);
```

For every pixel belonging to the tumour stroma, its coordinates were used to store simultaneously the distance from the closest vessels and its rhodamine signal intensity. The `label` option of the `bwdist` command allows to correlate the distance measured to the corresponding vessels, and therefore to retrieve its diameter. All data are stored in a matrix correlating pixel by pixel the rhodamine intensity, the distance from the closest vessel and the vessel diameter. The function `griddata` was used to produce a meshed grid of data useful to produce a surface plot of vessel diameter against distance, with the pixel intensity represented in a colour map (3.14-3.15):

```matlab
xlin=linspace(0,max_diam,max_diam*10);
ylin=linspace(0,max_dist,max_dist*10)';
zg=griddata(vess_diam,distance,intensity,xlin,ylin,'linear');
surf(zg,'Edgecolor','none')
```
Figure 3.14: Identification of vessels and branching points. From the transmitted light image (a) the full mask vessel was obtained (b). The skeleton mask (c) showing only the vessels central line was computed from the vessels mask. Finally, vessel ends (green), vessel branching points (red) and vessel centre (blue) were identified as reported in fig. 3.15. Bar 210µm.
Figure 3.15: Identifying vessels, ends and branching points in a binary mask. Pixels belonging to the middle of the vessel have sum of surrounding pixels of 2. Branching points have sum of 3 and ends have sum of 1.
3.7 Automatisation of image analysis

Automatisation of image analysis allows faster image processing and higher throughput. Image analysis automatisation was implemented with the development of two GUI softwares using MATLAB GUIDE toolbox. One software is dedicated to in vitro image analysis (3.17), the second one to in vivo image analysis (3.18). Both softwares store images in a database for batch processing and allow the user to categorise images using a tag system. The tag system is used to recall images from the database and to perform analysis on data sub-sets, i.e. pro-
cess only images acquired with a specific microscope or from cell treated with a specific polymersomes formulation. A panel allows to test different algorithm and post-processing mask settings in order to identify the ideal conditions for segmentations. The settings can be then applied to the entire database or to a sub-set specified by the tag system. Segmentation results can reviewed individually for further refinement. *In vitro* GUI was optimised to perform intensity based studies such as calculation of average intensity on specific ROIs, either in relation to specific growing condition (i.e. TGF-β1 concentration) or over time (i.e. uptake studies). The software can also perform co-localisation (Pearson and Manders coefficient). *In vivo* GUI was optimised to identify the three ROIs blood vessel, vessel edges and tumour stroma and to measure the relative average intensity.

![GUI of the software used to analyse ICC and Immunohystochemistry (IHC) images. The interface is divided in panels to group different stages of the analysis: data input, data retrieval, mask creation, combination of masks, analysis and plotting. On the right, the chosen picture is always displayed. During batch analysis, the right picture box provides feedbacks on the analysis status (i.e. on-going, finished, errors).](image)

Figure 3.17: GUI of the software used to analyse ICC and Immunohystochemistry (IHC) images. The interface is divided in panels to group different stages of the analysis: data input, data retrieval, mask creation, combination of masks, analysis and plotting. On the right, the chosen picture is always displayed. During batch analysis, the right picture box provides feedbacks on the analysis status (i.e. on-going, finished, errors).
Figure 3.18: GUI of the software used to analyse tumour vasculature images. The interface is divided in panels to group different stages of the analysis: data input, data retrieval, mask creation, analysis and plotting. On the left, the chosen picture is always displayed along with the different masks obtained.
CHAPTER 4

POLYMERSOME UP-TAKE

Abstract

Fluorescently labelled polymersomes can be detected in cells using a variety of techniques, including Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC), Fluorescence Activated Cell Sorting (FACS) and microscopy. Of these, the latter is the most sensitive, being capable of resolving uptake at the single cell level. It is also the quickest one to perform routinely in a high throughput screening context, as it does not involve extraction or preparation steps: cells can be imaged live. In this part of the work, the use of fluorescence microscopy to quantify cellular uptake was validated through comparison with consolidated quantitative (RP-HPLC) and semi-quantitative (FACS) techniques. Furthermore, the analytical system was complicated by the introduction of an in house developed flow bio-incubator to assess the effect of shear stress and fluid velocity on cellular uptake.

The polymersomes used had hydrophilic brush composed of Poly(2 - methacryloxyethyl - phosphorylcholine) (PMPC) or poly(oligo(ethylene glycol) methacrylate) (POEGMA), known to interact with cells in different ways: PMPC based polymersomes were expected to be internalised quickly, while POEGMA based polymersomes were expected to have a significant slower uptake kinetic. The tumour microenvironment was replicated in vitro by studying the uptake in three cell type characteristic of the disease: tumour cells (Mouse Fibrosarcoma Cells expressing VEGF188 isoform (MFC188)), blood vessel endothelial cells (Small Vessel Endothelial Cells (SVECs)), and perivascular cells (10T1/2). MFC188 cells can originate a more vascularised and pericytes rich tumour when used in vivo in Dorsal Skinfold Chamber (DSC) preparation (Tozer et al. [2008]). This will facilitate vascular imaging in vivo in later experiment presented in this thesis.
For this reason MFC188 cells were chosen as a model of tumour cell for both \textit{in vitro} and \textit{in vivo} studies.

4.1 Introduction

4.1.1 Uptake and \textit{in vitro} screening

A comprehensive understanding of the mechanism and parameters regulating uptake of polymersomes by cells is at the basis of a clever design of nanoparticles for a specific application.

Direct quantification of the vector up-taken \textit{in vitro} is strongly dependent on the properties of the vector itself. Delivery of radioactive marked nanocarriers can be quantified by measuring the radioactivity signal (Penate Medina et al. [2010]). This is a very sensitive technique and it is also useful to determine the metabolic fate of the carrier, however it is a costly technique and it requires specific equipment and working spaces. Fluorescently labelled carriers represent a valid alternative (McDonald and Choyke [2003], Beerling et al. [2011]) both \textit{in vitro} (Massignani et al. [2010]) and \textit{in vivo} (Christian et al. [2009a]), a routine step of pre-clinical development. Fluorescently labelled particles can be detected in a number of way. \textit{In vitro}, the most commonly used techniques for detection of fluorescently labelled vectors in cells are RP-HPLC, FACS and cell imaging.

**RP-HPLC** is a quantitative technique capable of separate solute according to their charge and molecular weight, and to quantify their concentration when coupled with a detector, typically a UV-VIS spectrophotometer or a fluorometer (Horvath et al. [1967], Gerber et al. [2004]). In drug development and discovery it has been used to detect a number of different molecules, from peptides to anti-cancer drug (Carr [2002], Tekade et al. [2013]). In this study, RP-HPLC was used to quantify the amount of polymersomes, specifically of rhodamine labelled colpolymer, up-taken by the cell.

**FACS** is a technique capable of sorting every cell in the cell individually on the basis of its size and granularity/complexity. As a result, FACS allows to separate (sort) different cell type in a sample as they will have different size and granularity (Dittrich and Gohde [1973], Kiziltepe et al. [2012], Lee and Tung [2011]). Furthermore, every cell is analysed for fluorescence intensity at a given wavelength. This aspect is of importance for this work as it allows to obtain information regarding the amount of rhodamine labelled polymersomes internalised by cells (Massignani et al. [2009]).
Live cell imaging, specifically coupled with fluorescence detectors, is a commonly used technique to assess cellular uptake of fluorescent labelled moieties. Traditionally capable of micrometer spatial resolution, it is now possible to break the diffraction limit and to resolve at the nanometer scale (Hell and Wichmann [1994]). Fluorescence microscopy can record intensity of emission from fluorescent material uptaken by cells, and the signal strength is proportional to the amount of material uptaken (Canton and Battaglia [2013], Kim et al. [2009], Discher et al. [2008]).

4.1.2 Static and flow in vitro cell growth

In vitro study of cells in every day laboratory practice is mostly performed on a plastic Micro well plate (MWP). MWPs are a confined environment fully controlled by the researcher, making them ideal to increase reproducibility of the analysis and to study impact of single variables on cellular phenomena. However, cells in the body are subjected to a much more complex environment given by interactions with other cells types, different chemicals and mechanical stimuli. One of the determinants of the local concentration of chemicals is blood flow, as it is responsible for mass transfer. Blood flow affects cells also because of direct mechanical stimuli, to the point that some cells have specifically evolved to sense flow and to adapt phenotype in response to variation of flow. An example of such cells include endothelial cells, the cells that constitute the wall of blood vessels. An interesting study on the effect of shear stress on endothelial cells (HUVEC) was performed by Khan and Sefton. Using a specifically designed microchip that recreates a number of obstructions and channels of different sizes they were able to recreate several different shear stress and flow streamlines within the same chip. They then investigated the changes in expression of VE-cadherin and other adhesion molecules to show a clear effect of the flow on the expression of such molecules and the phenotype of the cells (Khan and Sefton [2011]).

4.1.3 Effect of flow on cellular uptake

It is easy to speculate that flow can affect cellular uptake, especially by cells specialised to sense flow such as the endothelial cells. Despite this there is a surprisingly lack of literature supporting this statement, most uptake studies being still conducted in static conditions in MWP. A recent study performed by Volkov and co-workers correlates uptake of positively and negatively charged quantum dots and SiO$_2$-NPs by endothelial cells (HUVEC) under different shear stress stimulation. Surprisingly, they reported no uptake in static in 20 min despite one of the formulation being significantly toxic at the same exposure time. Fur-
thermore, they showed maximum uptake at low shear stress (0.05 Pa) for all the formulations tested, and then a decrease of up-take that reflects the increase in shear stress, even if the trend was not statistically relevant \cite{Samuel2012}. In another study Discher and co-workers investigated the effect of flow on up-take of filomicelles of different lengths. They reported how immobilised macrophages under flow rates similar to the ones measured in spleen \textit{in vivo} can recognise and uptake small spherical vesicles, but the flow forces the filomicelles in a streamlined conformation, and as a results a small fragment of the tip can be detached and up-taken, but the majority of the mass of the filaments is rapidly taken away from the cell by the flow therefore prolonging its circulation time in the device. This \textit{in vitro} data was confirmed in \textit{in vivo} results showing circulation half time proportional to filament length \cite{Geng2007}. To date, no studies have been published providing a comprehensive correlation of uptake for a given polymersomes formulation and cell line with shear stress.

### 4.1.4 Flow devices

Several devices have been designed to overcome static tissue culture limitations. Such devices tend to be application specific and custom made, not commercially available. Examples includes reproduction of blood vessels \cite{Miyakawa2008, Leong2013, Samuel2013}, heart valves \cite{Dumont2002}, liver \cite{Powers2002} and bones \cite{Kim2012}. An interesting exception is represented by the flow bio-incubator developed by Mazzei and collaborator, \cite{Mazzei2010}, and commercialised with the name \textit{Quasi-Vivo}® by Kirkstall, a company based in Sheffield. The system resembles a 24 well plate and has a modular nature which allows interconnecting of chambers to recreate cross-talking between organs, an approach they describe as \textit{organomics}.

An important classification of flow bio-incubator is based on sized. Most of the devices cited above have chambers at least millimetre-sized. Another popular type of devices is known as micro-fluidic devices. These have the great advantages of being highly reproducible because of the precise manufacturing process and to minimise the requirements of cells, media and other reagents. However, their small size is also their main limitation. Because of the limited number of cells used they cannot be representative of big and complex tissues \cite{Tingley2006}. Furthermore, because of their geometry they have a big edge/surface ratio \cite{Lundholt2003}.
4.1.5 Aim

RP-HPLC, FACS and live cell imaging are all techniques suitable for quantifying polymersomes uptake. In routine screening for uptake the technique giving the most accurate results should be used, and that technique should ideally be fast and easy to set-up. Here the three techniques were compared and integrated in the study of uptake of different formulations by different cell type. The aim was to validate the use of live cell microscopy as a semi-quantitative technique to screen polymersomes uptake by tumour-relevant cell types: tumour cells (Mouse Fibrosarcoma Cell (MFC)), endothelial cells (SVECs) and perivascular cell (10T1/2). Furthermore, the analysis protocols will be applied to in flow uptake studies, to elucidate the role of shear stress and fluid velocity on cellular uptake. This is critical as polymersomes will be administered in vivo Intra Venous (i.v.), therefore they will be subjected to flow dynamics.

4.2 Results

4.2.1 Static conditions

Quantification of uptake with RP-HPLC, FACS and live cell imaging

The technique of choice in this work is imaging, therefore almost all uptake data collected will be based on image analysis. Comparison with established analytical techniques RP-HPLC and FACS was used to validate the method. MFC188 cells were treated with 1mg/ml of rhodamine labelled Poly(2 - methacryloyethyl-phosphorylcholine)-poly( 2 -(diisopropylamino)ethyl methacrylate) (PMPC-PDPA) polymersomes and the uptake was quantified with RP-HPLC, FACS and live cell image analysis. All three methods showed very similar uptake profile (fig. 4.1).
4. Polymersome uptake

4.2. RESULTS

Figure 4.1: RP-HPLC, FACS and live cell imaging comparison. All uptake refer to 10% rhodamine labelled PMPC-PDPA polymersomes, 1mg/ml, MFC188 cells. All data were normalised with respect to the maximal uptake for the relative quantitative technique, corresponding to the uptake at $t=24h$. All techniques have similar uptake profile. $N=3$, ±SD.

Once confirmed the validity of the method, live cell imaging analysis was used to compare uptake of rhodamine labelled PMPC-PDPA and poly(oligo(ethylene glycol) methacrylate)-poly(2-(diisopropylamino)ethyl methacrylate) (POEGMA-PDPA) polymersomes from MFC188, SVECs and 10T1/2. All cells were treated for up to 24h with 1mg/ml of rhodamine labelled polymersomes. For any of the three cell types, uptake of PMPC-PDPA polymersomes was higher compared to POEGMA-PDPA ($p<0.05$) (fig. 4.2). This was observed both in FACS and image analysis. MFC188 had higher uptake compared to SVECs or 10T1/2, especially for PMPC-PDPA polymersomes.
4. Polymersome uptake

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Figure 4.2: Quantification of uptake of rhodamine labelled PMPC-PDPA and POEGMA-PDPA polymersome by 10T1/2, MFC188 and SVECs. Uptake was quantified by FACS (a) and image analysis (b). PMPC-PDPA uptake was greater than POEGMA-PDPA, and MFC188 had greater uptake compared to SVECs or 10T1/2, with the only exception of uptake of POEGMA-PDPA measured by FACS. N=3, ±SD.
4.2. RESULTS

Figure 4.3: Representative images of PMPC-PDPA uptake in live cells imaging from 10T1/2 (A, B, C, D), MFC188 (E, F, G, H) and SVECs (I, J, K, L). Images were acquired at 0h, 1h, 6h and 24h. Cells cultured in static conditions. Uptake in MFC188 is greater than uptake in 10T1/2 or SVECs. Bar 70µm.

Figure 4.4: Representative images of POEGMA-PDPA uptake in live cells imaging from 10T1/2 (A, B, C, D), MFC188 (E, F, G, H) and SVECs (I, J, K, L). Images were acquired at 0h, 1h, 6h and 24h. Cells cultured in static conditions. Uptake in MFC188 is greater than uptake in 10T1/2 or SVECs. Bar 70µm.

4.2.2 Flow bio-incubator

Flow bio-incubator optimisation

The flow bio-incubator developed here was based on the 24 well plate geometry for easy translation of existing experimental protocols to the new device.
COMSOL MultiPhysics was employed to assess shear stress at the cellular level and to optimise the design to deliver a homogeneous flow. From the first simulations run a clear problem emerged: the inlet and outlet positioned perpendicular to the cell plane generated a high local peak of shear stress. Furthermore, this design was difficult to assemble in a reproducible manner: the tubes were subjected to movement that deviated from the simulated perpendicular angle (see fig. 4.5).

To protect the cells from perpendicular flow a shield was introduced, so that the liquid was entering the well parallel to the cell surface. This was achieved using L-shaped elbow connectors attached to the tubing. The elbow connectors had several advantages. Their bulk volume substituted liquid volume in the well, reducing the amount of media needed. Furthermore, the inlet and the outlet shield provided stability to the tubing reducing movement. As with the previous design, the position of the tubing was optimised using in silico modelling. Results from the simulations are reported in figure 4.6-4.7. When inlet and outlet are too close to the cells (H=1mm) there is almost no flow at the cell level (velocity and shear stress close to 0). At medium high (H=4mm) the situation improved, and finally at H=7mm the flow was higher, but less parallel to the horizontal plane. Regarding the relative position of inlet and outlet it was found that if they are located close to the edge the flow profile was irregular with localised peaks of shear stress and velocity close to the wall edges. If the tubes are located close to the centre the profile was more homogeneous. Considering the in silico results, the model chosen was with inlet and outlet located at the edges of the well and at 4mm from the cell level. This model was the easiest to assemble in a controlled way, and it gave a good balance between homogeneous flow at the cellular level and shear stress-velocity magnitude range in relation to the peristaltic pump set-up. Positioning the tubing further away from the cells would have caused loss of accuracy at lower rpm and flow rate, which are the critical parameter to use when simulating low flow rates such as those characteristic of the tumour microenvironment.
Figure 4.5: Flow device models. The three proposed flow device models. The first model (top row) had no shield. The second and third models (second and bottom rows) had a shield orientating the flow parallel to the cell plane. The difference between the two models is in the relative position of the inlet and the outlet. In the second model they are close to the well wall, in the third model they are located close to each other. In silico simulations were run positioning the tubing at close to the bottom of the well (left column, Position 1), in the middle of the well (middle column, Position 2) or far from the cell level (right column, Position 3). Flow = 20 µL/s. * Identifies the inlet. Scale bar 7 mm.
Figure 4.6: Shear stress distribution across the three models. The coloured disc represent the plane of cell growth (bottom of the well). When no shield is present (top row) there is high heterogeneity between different areas of the well depending on their location with respect to the inlet or the outlet. When a shield was used (second and third rows) the shear stress distribution was more homogeneous. However, when the shield was too close to the bottom of the well (Position 1) there was almost no flow (shear stress $\approx 0$ dyn/cm$^2$ under the shield itself. Mean refers to the mean shear stress generated $5\mu$m from the bottom of the well. Flow= 20$\mu$L/s. * Identifies the inlet. Scale bar 7mm.
4. Polymersome up-take

4.2. RESULTS

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Figure 4.7: Velocity distribution across the three models. The velocity considered here is the total absolute velocity parallel to the cell plane. The coloured disc represent the plane of cell growth (bottom of the well). The general observations reported for shear stress in figure 4.6 are confirmed for the velocity profiles here presented. Mean refers to the mean velocity generated 5µm from the bottom of the well. Flow = 20µL/s. * Identifies the inlet. Scale bar 7mm.

Figure 4.8: Flow device overview. The wells are connected with 0.8mm bore tubing to a peristaltic pump. In (a) the elbow connectors are made visible by sectioning the well plate. In (b) the lid used is visible. The white circles are self-sealing membrane pierced with a 30G needle when injecting polymersomes into the system.
4. Polymersome uptake
4.2. RESULTS

Cell viability

Cell viability was measured as described previously (sec. 2.4.2), and cell viability of cells growing in static was compared to cell viability of cells growing under flow. The flow rate chosen for this analysis was 30µl/s because this value is 50% higher than the maximum value planned to be used in the uptake experiment, and it was assumed that if cells have no significant decrease in viability at this flow rate, then any lower flow rate could have been used without compromising cell viability. Cell viability was assessed for MFC188, SVECs and 10T1/2 treated with Transforming Growth Factor-β1 (TGF-β1). Results are reported in figure 4.9. No significant decrease in viability was observed for any of the cell lines (p>0.05).

![Graph showing cell viability comparison between MFC188, SVECs and 10T1/2.](image)

**Figure 4.9:** Cell viability in flow, (3 - (4,5 - Dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) assay. Cell viability was assessed for 10T1/2, MFC188 and SVECs. No significant decrease in cell viability was observed for any of the cell type used with respect of the same cell type cultured in static condition: 100% viability (U-test, p>0.05). Flow rate was 30µL/s. N=3, ±SD.

Cellular uptake

In this study we compared uptake at 20µl/s with uptake in no flow conditions. We compared PMPC-PDPA and POEGMA-PDPA, MFC188, SVECs and 10T1/2. Uptake was quantified by FACS and live cell image analysis. Results from both analysis showed higher uptake of polymersomes in static compared to in flow conditions. Furthermore, uptake of PMPC-PDPA polymersomes is higher than uptake of POEGMA-PDPA. Cell imaging allowed for better resolution, probably due to a better sensitivity. Analysis of cell imaging data revealed that in flow conditions the difference in uptake kinetics between the two formu-
lations is minimal compared to the difference of uptake in static conditions. For example, uptake of PMPC-PDPA at 24h from SVECs is double compared to the uptake of POEGMA-PDPA, while in flow there was no significant difference. It is also interesting to notice that the difference between different cell types is minimal in flow compared to static, where mfc188 showed the greater uptake and 10T1/2 the lowest. Image analysis and FACS results are reported in fig. 4.11. FACS data expressed as percentage of cells positive for rhodamine over time are reported in section 9.2, figure 9.5-9.7.
Figure 4.10: Uptake of PMPC-PDPA (b, f) and POEGMA-PDPA (d, h) from 10T1/2, MFC188 and SVECs in static and in flow (20µl/s). Images a, c, e, g are bright field images showing cells distributions in the well. Uptake in static conditions is greater than uptake in flow, and uptake of PMPC-PDPA is greater than uptake of POEGMA-PDPA. In static condition uptake from MFC188 is greater than uptake from SVECs or 10T1/2, while in flow conditions there is little difference between the three cell types. Bar 170µm.
4. Results

Figure 4.11: Quantification of uptake in static (solid lines) and in flow (dotted lines) conditions with FACS (a, c, e) and imaging (b, d, f). Uptake was quantified for PMPC-PDPA (red lines) and POEGMA-PDPA (blue lines) polymersomes. 10T1/2 (a, b), SVECs (c, d) and MFC188 (e, f) cells were compared. Uptake in static conditions is greater than uptake in flow, and uptake of PMPC-PDPA is greater than uptake of POEGMA-PDPA. In static condition uptake from MFC188 is greater than uptake from SVECs or 10T1/2, while in flow conditions there is little difference between the three cell types. N=3, ±SD.

Image analysis and well mapping

In the developed device, shear stress and velocity magnitude at the cell growth level is not perfectly homogeneous (see fig. 4.6-4.7). This is generally regarded as a disadvantage, especially compared to commercially available flow macro-chamber (i.e. Ibidi IV® chamber slides) or microfluidic devices. In those bioincubators the flow delivered is perfectly homogeneous across the whole observation chamber. However, in this study this can be used as an advantage. First of all, it can reproduce heterogeneous flow in tumour microenvironment, but it can
also be used to obtain uptake data in different flow conditions from the same well. In this situation the only variable is the flow rate at the cell level, as the rest of the well is exactly the same (same cells, same formulation, same pH and temperature). In order to correlate uptake with different areas of the well characterised by different shear stress, parallel (horizontal) or perpendicular (vertical) velocity the microscope was programmed to acquire images in different areas of the well, and the well was divided into eight regions having different flow properties. This was of interest because it was hypothesised that cells exposed to different flow rate, therefore to different shear stress and flow velocity, could not only exhibit altered phenotype, such as elongated shape as already reported for endothelial cells (Khan and Sefton [2011]), but also different uptake. The analytical method here reported will allow quantify uptake in relation to flow parameter in the same well, therefore in cells otherwise identical. This approach will also increase the throughput of the analysis by allowing multiple data collection (i.e. uptake vs flow) in the same well. The process of dividing the well into different areas characterised by different flow properties is described in section 3.5.2-3.5.3. Details of the different Regions of interest (ROI)s with corresponding physical properties are reported in table 4.1-4.3. This analysis was performed for PMPC-PDPA polymersomes up-taken by MFC188.
## 4. Polymersome uptake

### 4.2. RESULTS

<table>
<thead>
<tr>
<th>ROI</th>
<th>Shear stress range [dyn * cm(^{-2})]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10[\mu l/s]</td>
</tr>
<tr>
<td></td>
<td>0.00/0.75</td>
</tr>
<tr>
<td></td>
<td>0.75/1.50</td>
</tr>
<tr>
<td></td>
<td>1.60/2.25</td>
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<tr>
<td></td>
<td>2.26/3.00</td>
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<tr>
<td></td>
<td>3.10/3.75</td>
</tr>
<tr>
<td></td>
<td>3.76/4.60</td>
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<tr>
<td></td>
<td>4.70/5.25</td>
</tr>
<tr>
<td></td>
<td>5.26/6.00</td>
</tr>
</tbody>
</table>

Table 4.1: ROI segmentation for shear stress. Shear stress in dyn * cm\(^{-2}\). Data for flow rate of 10[\mu l * s\(^{-1}\)] and 20[\mu l * s\(^{-1}\)]. Data refers to division of ROI from Position 2, shield located near walls, reported in figure 4.6. The resulting well segmentation with all eight different ROI is reported.
### Table 4.2: ROI segmentation for horizontal velocity magnitude. Velocity in $\mu m \times s^{-1}$. Data for flow rate of $10 \mu l/s$ and $20 \mu l/s$. Data refers to division of ROI from Position 2, shield located near walls, reported in figure 4.7. The resulting well segmentation with all eight different ROI is reported.
4. Polymersome up-take

4.2. RESULTS

Table 4.3: ROI segmentation for vertical velocity magnitude. Velocity in $\mu$m s$^{-1}$. Data for flow rate of 10$\mu$l s$^{-1}$ and 20$\mu$l s$^{-1}$. Data refers to division of ROI from Position 2, shield located near walls, reported in figure 4.7. The resulting well segmentation with all eight different ROI is reported.

<table>
<thead>
<tr>
<th>ROI</th>
<th>10$\mu$l/s</th>
<th>20$\mu$l/s</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Velocity range [$\mu$m s$^{-1}$]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-6.00/-4.50</td>
<td>-12.00/-9.00</td>
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<tr>
<td></td>
<td>-4.99/-3.00</td>
<td>-8.99/-6.00</td>
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<td>-2.99/-1.50</td>
<td>-5.99/-3.00</td>
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<td>-1.49/0.00</td>
<td>-2.99/0.00</td>
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<td>0.01/1.50</td>
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<td>3.01/4.50</td>
<td>6.01/9.00</td>
</tr>
<tr>
<td></td>
<td>4.51/6.00</td>
<td>9.01/12.00</td>
</tr>
</tbody>
</table>

Images recorded by the epifluorescence BD-Pathway 855 microscope equipped with a 4X objective were analysed as described previously (sections 3.5.2-3.5.3) to correlate average fluorescence intensity with any specific flow parameter. Results are reported in figure 4.12-4.14. Analysis revealed higher uptake (p<0.05) in static compared than in flow, as already seen in FACS and single image analysis (section 4.11). Furthermore, it can be observed that uptake at 10$\mu$l s$^{-1}$ is higher than uptake at 20$\mu$l s$^{-1}$, particularly at the t=24h time point. However, the relative small difference seems to indicate that in the range of flow rates studied there is no significant difference of uptake (p>0.05): there is a significant difference compared to static conditions, but once flow is applied the difference is minimal across the range 10-20$\mu$l s$^{-1}$. Shear stress and horizontal velocity magnitude had a peak of uptake generally located in the middle of the well, corresponding to average values and more horizontal fluid streamline (see fig. 4.5). Vertical velocity showed a higher correlation of increased uptake with increased velocity magnitude, with higher uptake in areas with higher velocity.
Figure 4.12: Mapping of rhodamine intensity in the eight different ROI for shear stress. The average intensity measured in the area of a given ROI, identified as described in figure 3.10 is represented in colour code from blue (low intensity) to red (high intensity). Intensity is proportional to polymersomes uptake. Analysis refers to PMPC-PDPA polymersomes uptake by MFC188. In static condition (bottom row) uptake was homogeneous across the well. Uptake in flow at 20µl/s and 10µl/s was similar and uptake in the center of the well was greater than on the ROI corresponding to the inlet (white asterisk) and the outlet (black asterisk) region.
Figure 4.13: Mapping of rhodamine intensity in the eight different ROI for shear stress. The average intensity measured in the area of a given ROI, identified as described in figure 3.10 is represented in colour code from blue (low intensity) to red (high intensity). Intensity is proportional to polymersomes uptake. Analysis refers to PMPC-PDPA polymersomes uptake by MFC188. In static condition (bottom row) uptake was homogeneous across the well. Uptake in flow at 20µl/s and 10µl/s was similar and uptake in the center of the well was greater than on the ROI corresponding to the inlet (white asterisk) and the outlet (black asterisk) region.
4.3 Discussion

In this work, polymersomes will be engineered to image the tumour microenvironment. Before screening different formulations \textit{in vivo}, polymersomes were tested \textit{in vitro} for uptake kinetics in three tumour relevant cell types: tumour cells (MFC188), endothelial cells (SVECs) and perivascular cells (10T1/2). These three cell types were specifically chosen as they represent an in vitro model of the tumour that was implanted \textit{in vivo} experiments, obtained from MFC188 spheroid. This specific cell type is known to form tumours well vascularised and rich on perivascular cells (Akerman et al. [2013]). 10T1/2 are \textit{in vitro} precursors of perivascular cells.

Two different polymersome formulations were tested: PMPC-PDPA and POEGMA-PDPA. The two formulations chosen were expected to have different uptake kinetics, with PMPC-PDPA having faster uptake compared to POEGMA-PDPA.
The kinetic of uptake of fluorescent labelled polymersomes can be monitored using RP-HPLC, FACS and cell imaging. **RP-HPLC** and **FACS** are commonly used quantitative techniques, however they are less suitable to fast and sensitive screening than live cell imaging because of longer sample preparation. RP-HPLC is useful to quantify the exact amount of fluorescent labelled polymersomes delivered to the whole cell population. FACS analysis can sort cells in co-culture systems and analyse intensity at the single cell level. FACS cannot exactly quantify the amount of polymersomes delivered to the cells. Live cell imaging is the most sensitive technique as it allows to monitor uptake at the subcellular level. Like FACS, image analysis cannot quantify the exact amount of polymersomes up-taken, however it is a faster technique and it allows continuous monitoring of the same group of cells for the entire analysis, limiting the amount of materials required to perform the analysis. These characteristics make live cell imaging the ideal candidate for high throughput screening.

The image analysis method reported in section 3.4 was validated through comparison with RP-HPLC and FACS. The validation was performed studying uptake of 10% rhodamine labelled PMPC-PDPA polymersomes from MFC188. This formulation was chosen as it is known to have fast and high uptake from many cell type, especially cancer cells (Massignani et al. [2010], Pegoraro et al. [2014], Colley et al. [2014]). Results showed a very similar uptake profile in the time of analysis considered (up to 24h, fig. 4.1). Following experiments were therefore performed analysing uptake preferentially with optical imaging, and using FACS for confirmation. **RP-HPLC** was abandoned as it was considerably more time consuming and not suitable for high throughput set-up.

Uptake kinetics analysis showed a faster uptake for PMPC-PDPA compared to poly(2-(diisopropylamino)ethyl methacrylate) (PDPA), and a greater uptake from MFC188 compared to SVECs or 10T1/2. These findings are consistent with the reported mechanism of uptake of PMPC-PDPA, which is mediated by scavenger receptor SR-B1 (Colley et al. [2014]). POEGMA-PDPA uptake is not receptor mediated, therefore it was expected to have slower uptake. Furthermore, it has already been reported that PMPC-PDPA polymersomes have faster uptake compared to Polyethylen Oxide-poly(2-(diisopropylamino)ethyl methacrylate) (PEO-PDPA) polymersomes (Murdoch et al. [2010]). The hydrophilic block POEGMA used here is a chemically related to Polyethylene Oxide (PEO), as it is essentially made by short PEO chains linked to a polymeric backbone. Finally, the cited work of Colley and co-workers (Colley et al. [2014]) reported how tumour cells have greater expression of scavenger receptors compared to normal cells. Further experiments confirming the validity of the statement for MFC188 could explain the greater polymersomes uptake observed in this cell type com-
pared to SVECs and 10T1/2.

Uptake studies performed in static tissue culture conditions are a laboratory standard. However, cells in the natural environment are growing in completely different conditions, caused by different surrounding (i.e. other cell lines present in proximity), extracellular matrix and shear stress. This latter one can be mimic in vitro by using a flow bio-incubator. There are several flow chambers commercially available, however most of them are developed to mimic specific environments, such as coronary vessels, or to be used in conjunction with specific techniques, usually live cell imaging.

Considering that a number of protocol for RP-HPLC, FACS and live cell imaging analysis of uptake have been developed in the first part of the work, it was decided to develop a flow bio-incubator that allowed easy transfer of pre-existing protocols. The device was based on the geometry of 24 well plate. In silico modelling was used to optimise the design of the device so that the flow delivered was as uniform as possible and to make sure that the shear stress and flow rate delivered were comparable to the physiological ones at the capillary level: $\tau = 100 \text{dyn} \cdot \text{cm}^{-2}$, $U = 10 - 100 \mu \text{m} \cdot \text{s}^{-1}$ (Decuzzi and Ferrari [2006]). It should be noted that for the flow rates used in the final experiments (10-20 $\mu\text{l}/\text{s}$) the corresponding fluid velocity velocity was well within the indicated range, while the shear stress was $\simeq 8$ times lower. However, tumour microvasculature is characterised by irregular flow compared to healthy capillaries, therefore it could be that the stated value of $100 \text{dyn} \cdot \text{cm}^{-2}$ is lower in tumours. Furthermore, the experimental set-up used media, a Newtonian fluid with viscosity significantly lower than blood. Further development of the device will be focused on the use of more viscous media, for instance by adding dextran to the media.

Whole-well analysis of uptake kinetics were performed by FACS and cell imaging. The two analysis methods showed significant decrease of uptake for all cell lines under flow compared to static uptake. Furthermore, the difference of of uptake between the two polymersome formulations PMPC-PDPA and POEGMA-PDPA was significantly reduced. This could be explained considering that uptake is a dynamic event requiring interactions of polymersomes with specific cellular structures, such as scavenger receptors SR-B1 for PMPC-PDPA. When polymersomes are administered under flow, they are carried away from the cell surface by the flow. This is likely to reduce the interaction time between polymersomes and the receptors, and therefore to decrease the uptake rate. Furthermore, by limiting the receptor mediated uptake, the difference between PMPC-PDPA and POEGMA-PDPA observed in static are likely to be reduced consequently.

A close observation of the shear stress and velocity magnitude profile at the cell level (fig. 4.5-4.7) revealed areas characterised by different flow parameters.
This could be seen as a disadvantage of the device developed, which is unable to deliver perfectly homogeneous flow. However, live cell imaging unique capability to focus on different areas of the plate can be used to obtain uptake data of cells growing in slightly different conditions, but still on the same well. Potentially, this can considerably decrease the amount of materials and experiments needed to characterise uptake and flow correlation, with a positive impact on analysis throughput.

Cell growth area was divided in 8 different ROIs corresponding to 8 areas characterised by different horizontal or vertical velocity magnitude or shear stress. Fluorescence intensity of cells growing in one of the 8 ROI was use to map the uptake intensity in the area vs the corresponding physical parameter. For this analysis uptake in static (control) was compared to uptake at 10µl and 20µl. For the development and testing of the method the same combination of cell type and polymersome formulation used to validate imaging was chosen: MFC188 and 10% rhodamine labelled PMPC-PDPA.

Data showed a small but not significant (p>0.05) difference between uptake at 10µl·s\(^{-1}\) and 20µl·s\(^{-1}\). However, there is a clear decrease of uptake between no flow and 10µl·s\(^{-1}\). A comprehensive study on the effect of flow on nanoparticie uptake has not been reported yet. In 2012, Samuel and co-workers (Samuel et al. [2012]) reported a decrease in uptake of negatively charged quantum dots with increasing shear stress from 0.1Pa to 5dyn/cm\(^{-2}\), a behaviour similar to the findings here reported. However the two studies cannot be directly compared because of different experimental set-up. Samuel and coworkers used negatively charged quantum dots of 50nm of diameter, while we used 120nm neutral polymersomes. The uptake was studied for up to 20min after exposure in the cited work; in our experiment the uptake was analysed for 24h, with the first time point recorded after 30min. Finally, the average shear stress used in our system is up to 10 fold higher.

It should be noiced The inverse correlation between uptake rate and shear stress was confirmed in this work, however the two systems cannot be directly compared because of the different nanoparticles used: negatively charged 50nm quantum dots and non-charged 120nm polymersomes. Furthermore, in the cited work the uptake was monitored for 20min, in our experiment it was monitored up to 24h, with the earliest time point recorded after 30min.

Considering the segmentation of flow parameters, it was observed that for both shear stress and horizontal velocity magnitude there is an higher uptake in the region corresponding to the middle of the well, characterised by an intermediate parameter magnitude. This suggested a relatively low impact of shear stress or velocity on uptake in the range considered. Perhaps a wider range
would reveal a correlation between the physical parameter and uptake, but this correlation cannot be confirmed by the data reported. A correlation was observed between vertical velocity and uptake, with uptake higher at the higher vertical velocity (near the outlet) and lower at lower vertical velocity (near the inlet). A low uptake with low (negative, inlet) vertical velocity was expected, as it was the region were polymersomes were moving faster pushed away from the inlet. However, a high uptake at the outlet was not anticipated, as it is a region characterised by particles moving away from the cells towards the outlet of the chamber. Further investigation on polymersomes distribution in the well will clarify the role of vertical velocity and uptake, particularly with respect of local particles concentrations. Such results can be obtained by integrating the in silico simulation performed with the COMSOL Multiphysics CFD module with the module particle tracking.

In conclusion, different methods have been developed to assess polymersomes uptake under static and flow conditions. Protocols can be used to quickly screen several different formulations in combination with different cell types. Results obtained will be useful in the optimisation of polymersomes for imaging the tumour microenvironment, as it was found that PMPC-PDPA polymersomes are up-taken preferentially by tumour cells, while POEGMA-PDPA polymersomes have generally lower uptake, but less affected by flow dynamics in perivascular-like cells (10T1/2). Furthermore, PMPC-PDPA polymersomes fast uptake will likely correlate with fast clearance in vivo, while the other two formulations are expected to have longer circulation time in blood. This could result in an improved accumulation within the tumour via Enhanced Permeation and Retention (EPR) effect. Finally, uptake under flow conditions analysis revealed a strong effect of capillary-like flow rates on uptake, with significant reduction of uptake kinetic, especially for PMPC-PDPA. Further studies will better elucidate the correlation between uptake and different flow parameters, however preliminary results suggested that uptake by cells at the vascular and perivascular level will not be significantly shifted towards a specific cell line, as flow minimised the differences between cell types and polymersomes formulations.
Abstract

Dorsal Skinfold Chamber (DSC) allows detailed visualisation of tumour physiology in real time in vivo. Fluorescent labelled polymersomes have been successfully used in in vitro and in vivo imaging, however their use in a DSC chamber set-up has not been reported yet. The aim of this work is to assess the suitability of fluorescent labelled polymersomes for in vivo optical imaging using DSC. Poly(2-methacryloxyethyl-phosphorylcholine)-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA) and poly(oligo(ethylene glycol) methacrylate)-poly(2-(diisopropylamino)ethyl methacrylate) (POEGMA-PDPA) rhodamine labelled were injected Intra Venously (i.v.) into a mouse tail bearing a DSC. Preliminary results showed intravascular distribution of polymersomes at the early time point (1h) for both formulations, but at the later time point (15h) PMPC-PDPA was not detected in tumour vasculature or stroma, while POEGMA-PDPA was seen to extravasate and accumulate within the tumour. These finding will set the basis for future further development of polymersomes aimed at imaging the tumour microenvironment in vivo.

5.1 Introduction

5.1.1 Window chamber

In vivo optical imaging, and particularly high resolution Intra Vital Microscopy (IVM), is affected by poor tissue penetration of light. To negate this limitation the layers of tissues between the objective and the plans of focus can be removed and replaced with an optically transparent material. This approach
is adopted in the preparation known as the Window Chamber (WC). WC was first introduced in a rabbit model in 1924 (Sandison [1924]), adapted for mice in 1943 (Algire [1943]) and developed for application to the hamster cheek pouch in 1947 (Fulton et al. [1947]) before being optimised for angiogenesis studies in the mouse DSC in 1993 (Lehr et al. [1993]).

When applied to the back of the animal (DSC) a skin layer and associated connective tissue and fat are surgically removed from the dorsum to provide optical clarity. A chamber holds the skin away from the mouse body exposing the vascularised layer of muscle and skin on the opposite side of window. The preparation is then closed with a thin optical glass coverslip. Before closing the chamber, a tumour fragment or tumour cells are implanted (Koehl et al. [2009]). In a few days, the tumour starts to vascularise and angiogenesis can be imaged. Applications of IVM, an umbrella term including WC, have been extensively reviewed by Fukumura and co-workers (Jain et al. [2002]). Some of the most significant ones are reported in the next paragraph. The WC can be used to monitor molecular, cellular, anatomical and functional parameters. As Laschke defined, it is a 'window into the dynamic interaction of biomaterials with their surrounding host tissue' (Laschke et al. [2011]).

The WC preparation may be used to study a number of different parameters in vivo, at different levels of spatial resolution. Such parameters can be classified into molecular, cellular, anatomical and functional parameters.

**Molecular parameters** Molecular parameters include monitoring pH and pO$_2$, expression of receptors and molecular pathways (Helmlinger et al. [1997]). Monitoring such parameters require the use of probes that change optical properties according to the concentration of the molecule to test. An example of such probe that may be useful in measuring pH in vivo has been recently reported by Madsen and co-workers. They synthesised a copolymer linked to a dye that changes emission spectra at different pH values. This copolymer was used in the self-assembling of a class of nanocarriers known as polymersomes (Madsen et al. [2013]). Imaging in the WC also allows determination of the molecular mechanisms of angiogenesis. Fukumura and co-workers, using this technique observed that tumour cells induce production of Vascular Endothelial Growth Factor (VEGF) in stromal cells, which can represent up to 50% of the total VEGF present (Fukumura et al. [1998]).

**Cellular parameters** The WC can be used to observe the establishment and development of a tumour. A common strategy is to use genetically modified tumour cells expressing fluorescent proteins, mostly Green Fluorescent Protein
In vivo imaging 5.1. INTRODUCTION

(GFP). This allows the visualisation of tumour growth, to establish the minimal number of cells required for tumour growth and the visualisation of the tumour metastasis cascade: cells leave the primary site, extravasate into the blood vessels, reach a secondary site, extravasate into such location and finally establish as a secondary tumour (Chambers et al. [1995], Reeves et al. [2009]).

**Anatomical parameters** Anatomical parameters include vascular density, length, diameter and architecture. Such parameters are useful to describe the tumour microenvironment and to establish readouts for therapy. For instance, a decreased Micro Vessel Density (MVD) indicates the efficacy of an anti angiogenic agent, however MVD may not change if a reduction in vessel number is followed by a reduction in tumour size. Another anatomical feature derived from WC is pore-size cut-off, a critical aspect in nanocarrier therapy and imaging (Hobbs et al. [1998]). Nanocarriers extravasate to the tumour site through gaps between endothelial cells in the compromised vascular bed. To allow extravasation, it is therefore essential that nanoparticles have smaller radius than the inter-cellular gap. In the cited work, Jain and co-worker demonstrated the mechanism of extravasation, and they showed that extravasation of small molecules such as ($\approx 7\text{nm}$) is not affected in different tumours characterised by different gap (pore-size cut off) size, but in the range $200\text{nm}$-$1.2\text{µm}$. Opposite to albumin, extravasation of nanocarriers (liposomes or latex microsphere with diameter ranging from $250\text{nm}$ to $2.5\text{µm}$) was highly dependent on tumour type and localisation, with greater extravasation occurring in tumours with bigger gaps.

**Functional parameters** Functional parameters include hemodynamic variables such as red blood cell velocity, flow velocity and blood volume, vascular permeability and interstitial diffusion (Samuel et al. [2013], Øye et al. [2008], Dreher et al. [2006], Endrich et al. [1979], Reyes-Aldasoro et al. [2008]). Such studies are conducted using size-specific macromolecules, useful to evaluate extravasation and diffusion, or plasma markers such as the ones used in the study conducted by Reeves and co-workers (Reeves et al. [2012]) These studies elucidated not only the specific features of tumour vasculature compared to normal vasculature but also provided information related to the tumour heterogeneous blood supply and nanoparticle design to achieve better intra-tumour distribution.

DSC is also used in multi-modal imaging, normally combined with Magnetic Resonance Imaging (MRI). This approach is useful to validate vascular parameters extrapolated from MRI by using IVM, even if errors are possible due to different time acquisition points, a problem that could be overcome by simultaneous multi-modal data acquisition (Gaustad et al. [2008], Neeman et al. [2001],
Lin et al. [2008]). Validation of MRI functional information is critical because MRI is not quantitative, a correlation is required between contrast agent concentration and signal strength. Furthermore MRI is widely used in clinical practice, while WC cannot be used in this context. Multi-modal imaging can also provide information which are complementary such as whole body drug biodistribution (MRI) and cellular effect (DSC).

WC preparations can be analysed longitudinally, spatially and temporally and multiple parameters can be extrapolated from the same animal. As a result, the molecular, cellular, anatomical and functional parameters described above can be obtained simultaneously. Perhaps the most cited example is the study conducted by Fukumura and co-worker, in which they monitored gene expression, angiogenesis and physiological function of the tumour (Brown et al. [2001]). In another study Tozer and co-workers investigated tumour response to Vascular Disrupting Agent (VDA) combretastatin after implantation of fibrosarcoma cell-lines over-expressing different iso-form of VEGF. They correlated response to treatment with vessel length and density (anatomical parameter) and vasculature leakiness (functional parameter) (Tozer et al. [2008]). Quantification of response to treatment is another key application of WC. Classical therapy response is evaluated in terms of tumour size. However tumour shrinkage is not the only possible outcome of a therapy and in animal models is not always indicative of tumour response. In response to treatment tumours may become quiescent, therefore there is no change in size and no apparent response to the treatment. In both cases, changes in tumour size are slow, and there is the need to understand if a therapy is effective in a certain time frame. WC can help to identify molecular markers that respond rapidly to therapy which may help in predicting the outcome, or even more interesting, they could help in designing a personalised therapy based on prediction of outcome generated by the presence of such markers.

WC in mouse has many advantages: the multiple imaging of a single animal limits the number of animals required to achieve statistical significance at any given time-point. There is fast recovery from surgery and imaging analysis can commence 48h after surgery. However its execution requires dedicated training and skills. Furthermore the chamber is not always an orthoptic site for the tumour of interest and this explains why chambers have been created to be implanted in different parts of the body (e.g. cranial chamber and abdominal chamber (Coisne et al. [2013], Ritsma et al. [2013])). Furthermore, tumour growth is visualised in a time-frame of days/weeks, while human tumour development can take months or even years. Finally, the 3D growth of the tumour is restricted by the coverslip on at least one of the two sides of the frame, and this can cause artificially increased intra-tumour pressure which affects vasculature permeability (Staton
et al. [2009], Gaustad et al. [2008]). Despite this limitations, WC is a valuable tool in angiogenesis and tumour biology research, being used to unravel tumour angiogenic mechanisms at different levels.

Figure 5.1: Using a DSC it is possible to observe angiogenesis at different levels, such as the anatomical level (i.e. micro-vascular density), the cellular level (i.e. primary tumour growth and tumour metastasis), the functional level (i.e. quantification of the red blood cell velocity) and the molecular level (i.e. feedback on $pO_2$ and $pH$ of the tumour).

5.1.2 Vascular Endothelial Growth Factor

VEGF is a family of cytokines including four main members in humans: VEGF-A, VEGF-B, VEGF-C and VEGF-D. The most important in cancer angiogenesis and the first one discovered was VEGF-A, which is a potent pro-angiogenic factor and stimulates vasodilation and vessel permeability. VEGF-A is also a mitogen and a chemoattractant (Shibuya [2013]). VEGF-A binds to two receptors: VEGFR-1 and VEGFR-2. There is evidence that the binding affinity to VEGFR-1 is higher than to VEGFR-2. In early angiogenesis binding to VEGFR-2 is pro-angiogenic, whilst binding to VEGFR-1 suppress the angiogenic response (Sawano et al. [1996], Fong et al. [1995]).

VEGF-A mRNA is transcribed from 8 exons alternatively sliced resulting in different isoforms, the most common ones being VEGF120, VEGF164, VEGF188 in mouse, 1 amino acid longer in human. VEGF120 does not have a heparin binding site and is readily diffusible, VEGF188 has a heparin binding site, and binds strongly to proteoglycans in the Extracellular Matrix (ECM) and cell membrane. VEGF164 has intermediate properties (Ferrara [2004]). During development in the mouse model, it has been shown that the presence of VEGF164 is sufficient
to establish a functional vasculature, VEGF120 supports endothelial cell proliferation, but the vessels formed are hemorrhagic. VEGF188 is essential for the formation of a normal vasculature in 50% of animals (Stalmans et al. [2002]).

5.1.3 Single VEGF-A isoform producing tumours

Mouse fibrosarcoma cells expressing single isoforms of VEGF have been developed and tumours obtained from such cells have been analysed in a DSC. Tumour growth rate is independent of the isoform used but dependent on VEGF concentration (Yu et al. [2002]). Tumours expressing different specific isoforms of VEGF have specific characteristics. VEGF120 tumours are less well-vascularised (CD31 staining) than VEGF188 or VEGF164 tumours, and VEGF188 tumours have higher pericyte coverage (α-Smooth Muscle Actin (α-SMA) staining). VEGF120 tumours are more permeable to injected FITC-dextran 40KDa, and this is consistent with lower pericyte coverage. In terms of tumour growth, VEGF120 tumours show dilation of pre-existing vessels which become highly hemorrhagic. VEGF188 tumour growth have narrower non hemorrhagic vessels. VEGF120 established tumours are characterised by a vasculature limited to the periphery while VEGF188 tumours are completely vascularised. Wild type (all isoform expression) and VEGF164 tumours had intermediate properties (Akerman et al. [2013]).

5.1.4 Nanoparticles extravasation in tumour

Nanoparticles can extravasate and accumulate within tumours via the Enhanced Permeation and Retention (EPR) effect (see 1.4.2). Once blood-borne nanoparticles reach the tumour, extravasation and tissue penetration is a function of nanoparticles size, shape, charge, vascular morphology and physical properties. The effect of particle size and shape has been discussed in section 1.4.3. Here, the role of vascular characteristics will be discussed.

The tumour vasculature is highly heterogeneous, characterised by vessels of different diameter and anatomy, including capillaries, venules, arterioles, vascular shunts, anastomosis and blood channels without endothelial cells (Jain [1988]). Furthermore, the organisation of the vessels is not constant in space or time, with heterogeneous flow rates (Dudley [2012]). In addition, tumours are often characterised by a central necrotic area surrounded by a vascularised, growing area. The first challenge is the homogeneous delivery of nanoparticles to tumour via heterogeneous vasculature.

Once nanoparticles reach the tumour and distribute in the vasculature, the intra-tumour penetration is a function of two main parameters: diffusion and
convection. Diffusion $D$ is a slow process and is dependent on surface area of the vessel ($S$) and on the difference in concentration of the nanoparticles between the plasma and the tumour stroma ($C_P - C_S$). Finally, convection is proportional to blood leakage (and therefore to vessel surface area $S$), to the difference in osmotic pressure between plasma and tumour ($\pi_P - \pi_S$) and the difference in hydrostatic pressure ($p_P - p_S$) (Jain [2001]).

To be able to predict the intra-tumour distribution of nanoparticles is of great interest as it would help to optimise the design of the nanoparticle to achieve specific delivery via the EPR effect and higher tumour accumulation, which in turn lead to an improved therapeutic outcome or improved signal-to-noise ratio for an imaging application. In the last decade a great effort has been made to model tumour vasculature and nanoparticle extravasation \textit{in silico} (Frieboes et al. [2013], Stylianopoulos et al. [2012], Chou et al. [2013], Sinek et al. [2004]).

Unfortunately, tumour \textit{in silico} models are still far from perfect, due to the difficulties relating to accurate modelling of events occurring simultaneously at the vascular level ($\mu m$) and at the nanoparticle level ($nm$) and for the computational demand of running the simulation for biologically relevant time periods. For this reasons a number of simplifications have been introduced in every model, limiting their broader applicability to different scenarios (i.e. different tumour, with different vasculature characteristics). Experimental data will assist in developing the critical parameters regulating extravasation and accumulation and to perfect existing models.

\subsection*{5.1.5 Aim}

DSC allows highly detailed visualisation of tumour pathophysiology in real time \textit{in vivo}. Fluorescent labelled polymersomes have been successfully used in \textit{in vitro} and \textit{in vivo} imaging, however their use in a DSC chamber set-up has not yet been reported. The aim of this work is to assess the suitability of fluorescently labelled polymersomes for \textit{in vivo} optical imaging using DSC. To do so, PMPC-PDPA and POEGMA-PDPA rhodamine labelled polymersomes were injected and their distribution in the tumour microenvironment was monitored using fluorescence microscopy.

\section*{5.2 Results}

Tumour growth after \textbf{Mouse Fibrosarcoma Cells expressing VEGF188 isoform (MFC188)} spheroid implantation was monitored regularly to assess the extension of vascularisation and animal well-being. A typical time-frame of tumour develop-
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Tumour vasculature can be observed starting at day 4 after surgery (4% of total chamber area), and it is fully established around day 7 (70%). After this time the vasculature and tumour will start to overgrow the chamber. Imaging sessions were performed when the tumour was covering between 70% and 90% of the chamber, 8 days after surgery.

Figure 5.2: MFC188 tumour development from 24h after surgery to 7 days after surgery (d). Tumours are highlighted by the black dotted line. Picture (e) shows a chamber with no tumour growing, picture (f) shows a chamber with tumour established. Bar (a, b, c, d) 1.5mm; (e,f) 7mm.

Rhodamine labelled polymersomes were injected via the tail vein (Intra Venous (i.v.)) and a number of imaging sessions were conducted regularly from immediately after injection up to 18h after injection. Imaging settings such as...
fluorescence intensity were defined before injection so that no background noise was detected. Images were acquired using a confocal microscope equipped with a 10X objective. Under transmitted light, vessels are visible in black over a bright tumour stroma. In the rhodamine channel, vessels are highlighted by polymersomes and are seen in bright over dark tumour stroma (fig. 5.3).

Figure 5.3: DSC images, 10X confocal microscopy. Image acquired 5 min after i.v. injection of rhodamine labelled POEGMA-PDPA polymersomes. On the left (a) transmitted light picture, on the right (b) fluorescence pictures. Rhodamine labelled POEGMA-PDPA can be seen localised inside the blood vessels. Bar 230 µm.

Representative images for rhodamine labelled POEGMA-PDPA polymersomes can be seen in figure 5.3. The images were acquired 5 min after injection. A time-lapse for the same polymersomes formulation is reported in figure 5.4. Transmitted light images always show vessels as dark structures, acting as a reference image. In the rhodamine channel it is visible that initially vessels are highlighted as bright over dark stroma (2 min, fig. 5.4-b), but at a later time point vessels appeared in dark over brighter stroma (15 h, fig. 5.4-f). This suggests polymersome extravasation and accumulation within tumour.
Figure 5.4: MFC188 tumour microvasculature confocal images, 10X objective. At earlier time point (a-d) the tumour stroma is darker than vessels in the rhodamine channel (b, d), indicating circulating polymersomes. 24h after injection the tumour stroma is brighter than the vessels (f), suggesting intra-tumour accumulation. Bar 230µm.
Rhodamine labelled POEGMA-PDPA and PMPC-PDPA polymersome localisation were compared. At earlier time points, both PMPC-PDPA and POEGMA-PDPA were localised in vessels. Interestingly, at later time points the PMPC-PDPA signal was significantly weaker in the vasculature and the tumour stroma, suggesting that the formulation was rapidly cleared from the circulatory system (see fig. 5.5).
Figure 5.5: Confocal imaging comparison of POEGMA-PDPA and PMPC-PDPA, transmitted light (a-f) and rhodamine channels (g-l). 10min and 1h after injection both PMPC-PDPA and POEGMA-PDPA are localised in vessels (g, j, h, k). At 24h, PMPC-PDPA (l) is almost not detectable, while POEGMA-PDPA is clearly localised in tumour stroma (i). Bar 200µm.
All images were analysed as described in section 3.6 and results are reported in figure 5.6-5.7. Confocal image analysis confirmed the qualitative observation of increased accumulation of POEGMA-PDPA compared to PMPC-PDPA polymersomes (higher average intensity). Furthermore, for both formulations it was observed that at early time points, blood vessels contribute to the overall image intensity at a higher percentage than the tumour stroma, while at later time points the contribution is reversed. Edges have intermediate contribution between stroma and vessels. Thus, this analysis confirmed the qualitative observation of extravasation (decreasing vessel contribution) and accumulation within tumour (increasing stromal contribution).

Figure 5.6: PMPC-PDPA in vivo image analysis. Data are represented as overall mean intensity of rhodamine signal in the tumour area (right axis) overlaid with a break down of the relative contribution of vessels, edges and tumour stroma to the signal intensity (left axis, coloured bars). The overall image intensity showed a slight increase over time. Moving from t=1h to t=16h it was observed that polymersomes relative distribution in the vessels Regions of interest (ROI) decreased, while the intensity of the stroma ROI increased.
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Figure 5.7: **POEGMA-PDPA** in vivo image analysis. Data are represented as overall mean intensity of rhodamine signal in the tumour area (right axis) overlaid with a break down of the relative contribution of vessels, edges and tumour stroma to the signal intensity (left axis, coloured bars). The overall image intensity showed a slight increase over time. Moving from $t=1\text{h}$ to $t=16\text{h}$ it was observed that polymersomes relative distribution in the vessels ROI decreased, while the intensity of the stroma ROI increased. At $t=16\text{h}$ there was a clear inversion between the two intensity distributions, evidence of the extravasation observed in fig. 5.5.

Qualitative and quantitative confocal image analysis suggested polymersomes extravasation and accumulation in tumours. Further image analysis was carried out to gain insight into the dynamics of tumour extravasation. Confocal images were segmented to correlate the amount of extravasation against the distance travelled by the polymersomes and the diameter of the nearest vessel, here assumed to be the origin of travel of polymersomes. The analysis is discussed in section 3.6.2. The analysis revealed less extravasation of PMPC-PDPA compared to POEGMA-PDPA, as was already anticipated by the previous analysis (fig. 5.6-5.7). The analysis was performed also in pictures acquired with epifluorescence microscope equipped with a 2.5X or a 20X magnification lenses, however only confocal images analysis is reported here. This is due to the lack of spatial resolution in epifluorescence microscopy compared to confocal microscopy: masks identifying different ROI can potentially be less accurate. Furthermore, in epifluorescence the transmitted light channel and the rhodamine channel were not acquired simultaneously, therefore the translation of the mask created on the transmitted light micrograph to the rhodamine one is less accurated as it
is affected by mouse movements. Image analysis for epifluorescence pictures is reported in annex section 9.3. Analysis of POEGMA-PDPA extravasation profile interestingly reveals how extravasation is more intense at the level of bigger vessels (≃12µm), however higher distances are travelled from lower calibre vessels (≃6µm) (see fig. 5.8-5.9).

Figure 5.8: Extravasation analysis of POEGMA-PDPA polymersomes. Over-time (0min-16h) mapping of extravasation. For every pixel belonging to tumour stroma, the distance to the closest vessel and the diameter of the closest vessels were measured together with the rhodamine intensity. Data were then mashed to obtain a heathmap where rhodamine intensity is colour coded from blue (low intensity) to red (high intensity). Polymersomes extravasating from smaller vessels (5-10µm) travelled the greatest distance (≃15µm. Polymersomes extravasating from larger vessels (10-15µm) travelled a shorter distance, however their accumulation is greater (greater intensity).


Figure 5.9: Extravasation analysis of PMPC-PDPA polymersomes. For every pixel belonging to tumour stroma, the distance to the closest vessel and the diameter of the closest vessels were measured together with the rhodamine intensity. Data were then mashed to obtain a heatmap where rhodamine intensity is colour coded from blue (low intensity) to red (high intensity). Polymersomes extravasating from smaller vessels (5-10 µm) travelled the greatest distance (≃15 µm). Polymersomes extravasating from larger vessels (10-15 µm) travelled a shorter distance, however their accumulation is greater (greater intensity).

5.3 Discussion

High resolution IVM of the tumour microenvironment has been made possible by the use of a DSC. The DSC had the advantage of reducing the limitation of poor tissue penetration of light characteristic of optical imaging techniques. DSC models have the disadvantage of being usually non orthotopic and results can be hindered by increased intra-tumour pressure, due to growth constraints by the two glass cover slips of the chamber, however it is still a widely used model to study angiogenesis and vascular response to therapy.

The tumour implanted within the chamber was a fibrosarcoma, derived from Mouse Fibrosarcoma Cell (MFC) cell expressing VEGF188. This particular cell type was chosen over the MFC wild type or cell-lines expressing VEGF120 or VEGF164 because studies from our group reported that they are characterised by a more vascularised tumour with higher pericyte coverage (Akerman et al. [2013]). This is a more appropriate tumour to start developing polymersomes for IVM imaging, compared to the MFC expressing VEGF120, where there is a poor vasculature.

The results here discussed represent the outcome of a very limited number of animals, not sufficient to draw conclusions based on solid statistic. Overall, this part of the work should be considered a pilot experiment aimed to test the
analysis methodology described in chapter 3 and to investigate possible correlation between \textit{in vitro} uptake discussed in chapter 4 and \textit{in vivo} intra-tumour distribution.

It was of interest to test both formulations \textit{in vivo} to visualise any differential distributions within the tumour microenvironment. Because of the greater uptake of PMPC-PDPA compared to POEGMA-PDPA polymersomes observed from the \textit{in vitro}, we expected to observe a shorter plasma half-life of PMPC-PDPA compared to POEGMA-PDPA polymersomes. It was also hypothesised that circulation time affects accumulation within the tumour and that the two formulations may have different intra-tumour distribution due to the interactions with different cell types in the tumour microenvironment.

Confocal microscopy allowed simultaneous acquisition from the rhodamine and transmitted light channel. Simultaneous acquisition is critical to transfer segmentation result from the transmitted light image to the rhodamine channel avoiding offsets generated by mouse movement. Qualitative image observation revealed gradual polymersomes extravasation and accumulation within tumour. Qualitative comparison between POEGMA-PDPA and PMPC-PDPA polymersomes images revealed that accumulation of POEGMA-PDPA was higher than PMPC-PDPA. This was confirmed by image analysis, performed using a specifically developed segmentation and analysis algorithm (section 3.6). POEGMA-PDPA distribution profile could be explained when considering the chemical similarity between poly(oligo(ethylene glycol) methacrylate) (POEGMA) and Polyethylene Oxide (PEO). PEGylated systems are known to have prolonged plasma half-life (Pasut and Veronese [2012]) because of their ability to avoid interaction with protein (\textit{non-fouling}) and to escape the Reticuloendothelial System (RES) (\textit{stealth} properties). POEGMA is likely to exhibit similar properties to PEO.

Finally, we investigated polymersome extravasation, specifically the relationship between distance travelled by the polymersomes over time with respect to the diameter of the closest blood vessel, here assumed to be the origin of diffusion. Despite having an overall different magnitude of accumulation, PMPC-PDPA and POEGMA-PDPA polymersomes showed similar diffusion profiles. A peak of extravasation can be observed at the level of largest vessels (>10\(\mu\)m), however the longest distance is travelled when extravasation occurs at the level of smaller vessels (<10\(\mu\)m) (fig. 5.5). A similar irregular accumulation has been reported by Yuan and co-workers for liposomes. Liposomes accumulated close to larger vessels, and normally at the bifurcation points (Yuan et al. [1994]). Higher accumulation around bigger vessels could be explained by the increased superficial area of larger diameter vessels (therefore increasing both diffusion and
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Convection), and perhaps increased hydrostatic pressure between larger vessels and tumour stroma, compared to smaller vessels and tumour stroma. This could be due to increased blood flow which in turn increases convection, which is a faster phenomena compared to simple diffusion. Furthermore, local rheological properties of flow at bifurcation (i.e. increased turbulence) could affect extravasation. Further development of the method should implement functional and cellular parameters. Functional parameters include hydrostatic and osmotic pressure and flow velocity, cellular parameters include macrophage distribution, which could be responsible for polymersomes uptake and local accumulation.

Overall, the findings suggest that PMPC-PDPA polymersomes rapidly interact with other cells and organs, including the RES which is the usual body defence mechanism against circulating non-self, such as the polymersomes. POEGMA-PDPA polymersomes have reduced interactions with the RES and therefore have prolonged half-life, resulting in the increased potential to extravasate into the tumour via the EPR effect. These hypotheses are supported by unpublished data from Prof. Battaglia, showing significantly shorter half-life of PMPC-PDPA compared to POEGMA-PDPA in mouse model. The reduced plasma half-life of PMPC-PDPA affects intra-tumour accumulation, which resulted in significantly lower accumulation compared to POEGMA-PDPA polymersomes. Distribution in other organs was not investigated in this work, however preliminary data obtained from analysis of biodistribution of PMPC-PDPA and POEGMA-PDPA in healthy SCID mouse were conducted previously by Prof. Battaglia group. For both polymersom formulations it was found a profile of biodistribution typical for nanoparticles, with significant accumulation in liver, spleen, lung and kidney. Further work with tumour bearing animals should be performed to investigate the overall intra-tumour accumulation compared to accumulation in other, off-target organs.

A final remark should be made on the surgical procedure. The rate of success proved to be poor, with major complications due to the tumour not vascularising in the chamber or the chamber itself falling on the flank of the mouse a few hours after surgery instead of staying on the top of the back. Some of these complications are currently being addressed by standardising the cell spheroid preparation or by changing some of the components of the chambers, such as the spacers between the two frames. However, more work is required to improve the outcome of the surgery, which has not only ethical implications, but is also vital to obtain reliable information from in vivo experimentation.
CHAPTER 6

PERIVASCULAR CELLS TARGETING

Abstract

In the previous chapters, methodologies for quantification of cellular uptake of polymersomes have been developed. In this chapter the methodologies developed will be integrated in the context of further development of functionalised polymersomes to target a specific cell type: perivascular cells. Perivascular cells is a collective name identifying a group of cells, namely pericytes and Vascular Smooth Muscle cells (vSMCs), in direct contact with endothelial cells on the outer side of the blood vessel, hence the name perivascular. In tumour angiogenesis the perivascular cells coverage is reduced and vasculature becomes unstable. The ability to image perivascular cells in vivo and to monitor their response to anti-angiogenic therapies may lead to an improved understanding of their role in angiogenesis. Active targeting of perivascular cells with polymersomes requires the identification of a suitable specific marker. The first part of this chapter will focus on the identification of such a marker in a in vitro cell model: 10T1/2 cells. The expression of such markers in 10T1/2 and other tumour micronvironmental cells will be compared: endothelial cells (Small Vessel Endothelial Cells (SVECs)) and cancer cells (Mouse Fibrosarcoma Cells expressing VEGF188 isoform (MFC188)). Finally, different synthetic strategies to functionalise polymersomes with a targeting antibody will be investigated and the functionalised formulation will be tested in vitro.
6.1 Introduction

6.1.1 Perivascular cells

Blood vessels are formed by the endothelial cells and a number of supporting cells collectively referred to as perivascular cells or mural cells. As the name implies, perivascular cells are located outside the blood vessels and in contact with the endothelial cells. The first report of perivascular cells was made by Rouget in 1873 (Rouget [1873]). He described contractile cells located outside and around small blood vessels (Rouget [1873]). Pericytes were initially called Rouget cells. A few years later Zimmermann renamed the cells as pericytes due to their perivascular location (Zimmermann [1923]). Since the discover of pericytes and the improvement in phenotyping and microscopy techniques, other perivascular cells have been reported, namely vSMCs. The main difference between the cell type is in markers expression and location with respect of the vasculature bed, i.e. in contact with capillaries, pre- or post-capillaries or larger vessels. However, there is no clear differentiation between the cell types, to the point that the name pericytes is often used as synonym for perivascular cells.

Perivascular cells are rounded cells with long primary cellular protrusions running parallel to the blood vessel, from which small secondary processes perpendicular to the blood vessel are observed. Where the secondary processes are in contact with the endothelial cell, a discontinuity of the basal membrane is observed, suggesting direct contact between the two cell types through peg-socket junctions and tight junctions. The ratio between the number of perivascular cells and endothelial cells is known as pericyte coverage, the term reflecting the lack of clear distinction between pericytes and other perivascular cells. Pericyte coverage varies in different organs with specific tissue characteristics. Tissues with high endothelial cell turnover have lower pericyte coverage and tissues subjected to high orthostatic blood pressure have high pericyte coverage (Armulik et al. [2011], Díaz-Flores et al. [2009]).

Pericytes are traditionally restricted to a capillary and post-capillary location, however other mural cells are found in the same area. Furthermore, it has been reported that the pericyte embryological origin differs between organs. Pericytes in the Central Nervous System (CNS) and cardiac tract vessels originate from the neural crest (Creazzo et al. [1998], Ema et al. [2003]) while in the rest of the body pericytes are mesenchymal in origin, with Transforming Growth Factor-β1 (TGF-β1) responsible for their differentiation (Creazzo et al. [1998]). In adult tissues, pericyte development is not well characterised. They have been reported to derive from tissue derived stem cells including pericytes themselves and myofibroblasts (Diaz-Flores et al. [1992]) but also from peripheral blood pluripotent stem cells.
6. Perivascular Cells Targeting

6.1. INTRODUCTION

Figure 6.1: A perivascular cell (P) in a capillary. Endothelial cells (EC) constitute the capillary tube, one perivascular cell is directly in contact with many endothelial cells and embedded within the basal membrane (BM). Red blood cells are pictured as well (RBC).

(Rajantie et al. [2004], Ozerdem et al. [2005]). In fact pericytes are both descendant and precursor cells and they have mesenchymal potential (Crisan et al. [2008], Crisan et al. [2009]). Pericyte identification is made difficult by the lack of a specific marker. Commonly pericytes are described as α-Smooth Muscle Actin (α-SMA), Platelet Derived Growth Factor-B (PDGF-B) and Nerve Glial antigen 2 (NG2) positive (Bergers and Song [2005]). Furthermore, they can be identified by assessing their position with respect of blood vessel using techniques such as Transmission Electron Microscopy (TEM) or Scanning Electron Microscopy (SEM) imaging (Wagner and Hossler [1992], Hayden et al. [2007]).

Perivascular cells functions

Due to their important position and structural role in the vasculature, perivascular cells contribute to the regulation of several homeostatic functions; including regulation of blood flow, stabilisation of blood vessels, vascular permeability, production of extracellular matrix and immune defence (Díaz-Flores et al. [2009], Bergers and Song [2005]).

Contractility

Contractile properties of perivascular cells have been described since the early original publications (Rouget [1873], Zimmermann [1923]) and are now well documented (Kutcher et al. [2007], Peppiatt et al. [2006]). Endothelin 1 secreted by endothelial cells stimulates pericyte contraction (Rucker et al. [2000]), whilst relaxation is induced by nitric oxide, atrial natriuretic peptide, adenosine, lipopolysaccharide and reactive oxygen metabolites (Edelman et al. [2006a], Edelman et al. [2006b]). The contraction mechanism is supported by the expression of the contractile protein α-SMA, a marker for both pericyte and vSMCs, which can also be transferred to endothelial cells through adherent junctions (Hirschi
and D’Amore [1996]). Contraction of perivascular cells in response to different stimuli results in hemodynamic regulation at the microvascular level, with the greatest impact observed in arterioles.

**Capillary permeability regulation** Perivascular cells regulate capillary permeability by two mechanisms: one physical and one chemical. Physical regulation is a consequence of the pericyte coverage. The amount of pericyte coverage on the albuminal side of endothelial cells correlates with capillary permeability (Hellstroem et al. [2001], Wang et al. [2012]). Perivascular cells are also sensitive to chemical signalling. In angiogenesis perivascular cells produce Vascular Endothelial Growth Factor (VEGF), known to increase blood vessel permeability (Reinmuth et al. [2001]). Although the mechanisms that regulate capillary permeability are not fully characterised it is generally accepted that perivascular modulate vascular permeability by modulating Endothelial cell (EC) signalling pathways.

**Vessel stabilisation** Vessel stability is the result of functional endothelial cell/endothelial cell, endothelial cell/perivascular cell and cell/Extracellular Matrix (ECM) contacts. This is achieved by a balanced secretion of factors such as TGF-β1, Platelet Derived Growth Factor (PDGF) and angiopoietin 1/angiopoietin 2 from both endothelial cells and perivascular cells (Armulik et al. [2005]).

TGF-β1 is a cytokine regulating cell proliferation and apoptosis modifying angiogenesis in different ways (Mehta and Dhalla [2013]). It is a potent activator of endothelial cell proliferation and induces mural cell recruitment with subsequent differentiation into pericytes. Mouse models with abnormal TGF-β1 signalling are embryonic lethal, demonstrating an unformed and non-functional vasculature phenotype (Larsson et al. [2001], Dickson et al. [1995]). TGF-β1 recruits and induces vSMCs differentiation which in turn promotes vessel stability. In wound healing TGF-β1 interacts with endoglin, a part of the TGF-β1 receptor complex expressed many proliferating and stem cells, in addition to promote vSMCs/pericytes differentiation(Valluru et al. [2011]). TGF-β1 is secreted in a latent form by both endothelial cells and perivascular cells, with contact between the two cell types being reported to be essential for TGF-β1 activation (Hirschi et al. [1997], Hirschi et al. [1998], Hirschi et al. [1999]). In the presence of TGF-β1, perivascular cells increase the production of α-SMA, whereas absence results in increased NG2 and desmin (Song et al. [2005]). Furthermore, TGF-β1 in conjunction with angiopoietin 1 decreases the production of PDGF-B by endothelial cells (Betsholtz [2004]).

The angiopoietin-Tie2 balance is another key regulator of vessel stability. An
increase in angiopoietin 2 expression is a marker of tumour angiogenesis (Zhang et al. [2003]), although neither the angiopoietin ligand or receptor expression is correlated as a prognostic indicator (Staton et al. [2011]). Angiopoietin 1 is secreted by perivascular cells, with the receptor Tie2 expressed by endothelial cells (Sundberg et al. [2002], Sato et al. [1995]). Angiopoietin 1 stabilises blood vessels by inhibiting endothelial cell proliferation. Angiopoietin 1 or Tie2 negative mice do not survive due to vascular abnormalities. It has also been hypothesised that endothelial cell deficiency induced by the absence of angiopoietin 1 also negatively affects TGF-β1 expression (Suri et al. [1996]). Angiopoietin 2 is an antagonist of angiopoietin 1 secreted by perivascular cells. Angiopoietin 2 over-expression has been reported to mimic angiopoietin 1 deficiency (Gale et al. [2002]). Angiopoietins require TGF-β1 to function. Angiopoietin 1 or angiopoietin 2 have no effect on endothelial cell proliferation and tube formation in vitro but angiopoietin 1 antagonises TGF-β1-induced tube disruption and angiopoietin 2 antagonises angiopoietin 1 (Ramsauer and D’Amore [2007]).

The PDGF-B/Platelet Derived Growth Factor Receptor-β (PDGFR-β) system is also required for vessel stabilisation. In angiogenesis PDGF-B is secreted by endothelial cells, particularly by the tip cell in a vessel sprout. The tip cell is the endothelial cell leading the growth of the forming vessel in angiogenesis. It is followed by other endothelial cells that will constitute the wall of the vessel and by perivascular cells that stabilise the forming tubular structure. PDGF-B is a chemotactic factor for perivascular cells (Bjarnegard et al. [2004]). Lack of PDGF-B or PDGFR-β is lethal in knock-out mouse models. The phenotype is characterised by reduced perivascular cells coverage which leads to a non-functional vasculature (Levén et al. [1994], Soriano [1994]).

VEGF is probably the most potent pro-angiogenic factor modulating both perivascular cells and endothelial function. It is responsible for direct stimulation of perivascular cells proliferation and migration and also to indirect stimulation of recruitment, by increasing NO production in endothelial cells. VEGF inhibition has proven anti-angiogenic effects by increasing pericyte coverage (Tozer et al. [2008]).

**Perivascular cells in angiogenesis**

Perivascular cells in the quiescent vasculature have different morphology, location and protein expression when compared to those during active angiogenesis. In the early stages of angiogenesis, perivascular cells bulge and the cytoplasmatic processes become shorter (Baluk et al. [2003], Ozawa et al. [2005]). The basal membrane is degraded, perivascular cells detach from endothelial cells and coverage is decreased, a process mediated by VEGF (Diaz-Flores et al. [2009]). In
the sprouting phase of angiogenesis, endothelial cells migrate in the direction of growth of the new vessels without perivascular cells recruitment (Reynolds et al. [2000], Bjarnegard et al. [2004]). Perivascular cells are normally recruited soon after and are among the first cells to colonise the new vascularised area. Other studies have shown that perivascular cells can lead endothelial cells in the vessel sprouting and have the ability to form functional tubes without endothelial cell recruitment (Ozerdem and Stallcup [2003]). If angiogenesis resolves, endothelial PDGF-B recruits perivascular cells thus increasing pericyte coverage. This is a stabilising event, but pericyte coverage alone is not sufficient to protect vessels from VEGF-induced degradation: endothelial cell/perivascular cell functional contact must be established to ensure full vascular stability through the mechanism previously described (see paragraph 6.1.1) (Hoffmann et al. [2005]). Finally, perivascular cells have been reported to influence metastasis, with perivascular cells function directly correlating to increased metastatic potential in a fibrosarcoma model, which may be mediated by increasing vasculature permeability (Xian et al. [2006]).

6.1.2 Perivascular cells in vitro models

Perivascular cells are an elusive cell population characterised by highly heterogeneous marker expression. Identification of perivascular cells in ex vivo studies relies mostly on the description of their location with respect to blood vessels, coupled with the expression of a panel of markers, none of which are expressed only or by all perivascular cells. In vitro studies have shown similar limitations, therefore there is not a unique model for perivascular cells in vitro. In some studies perivascular cells have been extracted from in vivo tissues (Bryan and D’Amore [2008]). Typically this is achieved in larger animals and examples include extraction from corpus luteum and the bovine retina (Beckman et al. [2006], Dore-Duffy [2003]). Extraction from smaller animals has been reported, including hamster skeletal muscle and stria vascularis of the mouse ear (Mogensen et al. [2011], Neng et al. [2013]). There is a commercially available source of perivascular cells extracted from normal fetal brain (Bagley et al. [2005]). Primary perivascular cells present the advantage of demonstrating similar characteristics to in vivo perivascular cells. However, extraction procedures are time consuming, costly and require specific equipment and training, although improvements with semi-automated systems and protocols are now available (i.e. gentleMACS from Miltenyi Biotec). Alternatively, perivascular cells can be derived in vitro using a stem cell precursor. Perhaps the most studied is C3H/10T1/2, Clone 8, commonly referred to as 10T1/2. These cells were isolated in 1973 from C3H mouse embryos.
Figure 6.2: Pericytes in angiogenesis. A stable vessel is characterised by endothelial cells (EC, red constituents of the tubes) associated with pericytes (PC, pink perivascular cell) and the structure is enclosed in the basal membrane (ECM, light blue layer). In response to pro-angiogenic signalling such as NO and VEGF the membrane is degraded and vessels become more permeable. Angiopoietin 2 then causes the dissociation of pericytes from the endothelial cells and vascular regression. PDGF-B signalling recruits pericytes which follow the tip endothelial cell. If angiogenesis resolve pericytes are recruited and make contact with the endothelial cell. Angiopoietin 1 stabilises the formed vessels. The deposition of a new ECM is promoted by TGF-β1.
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In 1997 D’Amore and co-workers hypothesised the use of 10T1/2 as perivascular cells precursors (Hirschi et al. [1997]) and in 1998 they reported a more detailed characterisation of such a system (Hirschi et al. [1998]). Cells responded in co-culture with endothelial cells, by directional migration following PDGF-B signalling, with co-culture increasing expression of the marker \( \alpha \)-SMA. In cell culture, TGF-\( \beta \)1 affected the phenotype of 10T1/2 and increased the expression of \( \alpha \)-SMA while decreasing the expression of NG2 (Darland and D’Amore [2001]). Recently, 10T1/2 cells have been used to recreate the vasculature not only in vitro, but also in vivo. A gel containing both endothelial cells and 10T1/2 as supporting cells has been implanted into the mouse cranial window chamber and a functional vasculature was developed (Samuel et al. [2013]).

The panel of markers identified by D’Amore and co-workers does not describe specifically pericytes or vSMCs cells. In fact, the authors referred to 10T1/2 as both pericytes and vSMCs precursor. This is due in part to the fact that there is no clear distinction between the two cell lines, at least regarding cellular marker expression. If the only differentiation possible is based on location (capillaries for pericytes, larger vessels for vSMCs), then of course an in vitro model as the one described above cannot distinguish between the two. In the work here presented, 10T1/2 were chosen as model for perivascular cells to investigate any selective targeting against tumour cells or endothelial cells, without the specific need to distinguish between pericyte or vSMCs.

6.1.3 TGF-\( \beta \)1 and perivascular cells

TGF-\( \beta \)1 has been reported to act as both a pro- and anti-angiogenic factor in vivo (Pepper et al. [1993]). This dual role can be explained in terms of the local concentration of TGF-\( \beta \)1. For example, specific concentrations (0.1ng/ml) appear more efficient in stimulating Smooth muscle cells (SMC) proliferation in vitro than lower (0.02ng/ml) or higher concentrations (1-5ng/ml) (Battegay et al. [1990]). TGF-\( \beta \)1 response is dependent on the expression of the Activin receptor-like kinase 1 (ALK-1), a receptor phosphorylated by TGF-\( \beta \)1 (Tallquist et al. [2003]). TGF-\( \beta \)1 is secreted by both endothelial cells and perivascular cells in an inactive form and the association between the two cell types is required for TGF-\( \beta \)1 activation, mediated by a mechanism involving gap junctions (Antonelli-Orlidge et al. [1989], Hirschi et al. [2003]). TGF-\( \beta \)1 also has the ability to modify specific perivascular markers expression. \( \alpha \)-SMA expression has been detected in perivascular cells treated with TGF-\( \beta \)1 but not in untreated cells, which in turn expressed NG2 and desmin.

The TGF-\( \beta \)1 response by 10T1/2 has mainly been studied in co-culture sys-
tems with endothelial cells, with tubular structures characterised. Selective blocking of TGF-β1 receptor resulted in reduced formation of tubular structures, suggesting a key role for TGF-β1 in recruitment and differentiation of 10T1/2 into perivascular-like cells (Darland and D’Amore [2001]). In single cell culture it has been reported that TGF-β1 increases the expression of α-SMA (Lien et al. [2006]).

**Perivascular cells markers**

A number of markers have been used to identify perivascular cells. However, none has been reported to be consistently expressed. Commonly used perivascular cells markers include PDGFR-β, NG2, Regulator of G-protein Signalling 5 (RGS5), α-SMA, desmin, aminopeptidase A and N and the reporter XlacZ4. Unfortunately the expression of such markers is highly heterogeneous. Variations have been reported not only between species, but also within organs in the same species. Furthermore, perivascular cells markers can be modified in pathological states (Armulik et al. [2005], Armulik et al. [2011]). As an example, α-SMA expression is not detected in normal skin and the CNS, but it is highly expressed in tumours implanted in these organs (Abramsson et al. [2002]). RGS5 expression follows a similar pattern in a mouse model of pancreatic islet cell carcinogenesis (Berger et al. [2005]).

α-SMA is part of the contractile apparatus of perivascular cells and is organised into cytoplasm microfilaments. α-SMA is expressed by perivascular cells in pre and post capillary vessels, but not in mid-capillary vessels (Nehls and Drenckhahn [1991]). α-SMA is not perivascular cells specific being expressed by other cells, such as fibroblasts and keratinocytes (Hinz et al. [2001], Szentmáry et al. [2013]). In 10T1/2 α-SMA expression has been reported to be up-regulated by exposure to TGF-β1 (Hirschi et al. [1998]). α-SMA expression is correlated to contractile properties of perivascular cells and is the basis for the molecular mechanisms regulating microvasculature flow and vessel diameter.

**PDGFR-β** is one isoform of the receptor for PDGF-B, secreted by endothelial cells and some tumour cells and responsible for perivascular cells recruitment. PDGFR-β is a tyrosine kinase transmembrane receptor and one of the members of the Platelet Derived Growth Factor Receptor (PDGFR) family. PDGF exists in 4 isoforms: A, B, C and D, which dimerise to PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD. PDGFR exists in two isoforms: Platelet Derived Growth Factor Receptor-α (PDGFR-α) and PDGFR-β, which dimerise after binding to PDGFR-αα, PDGFR-ββ and PDGFR-αβ. The receptor is characterised by two
intracellular domains responsible for the signal transduction of the signal and the five extracellular domains (Shim et al. [2010]). Affinity of the different receptor isoforms is reported in figure 6.3. PDGFR-β has been often reported to be expressed by 10T1/2 (Hirschi et al. [1997], Hirschi et al. [1998], Díaz-Flores et al. [2009]).

NG2 or High Molecular Weight Melanoma Associated Antigen (HMWMAA) is a chondroitin sulfate proteoglycan membrane receptor. NG2 was initially found to be expressed by immature cells in the CNS that demonstrated the ability to differentiate either into neurons or glial cells, thus the name nerve/glial antigen. NG2 is expressed by several cell-types including mesenchymal stem cells and perivascular cells, both in physiological and pathological angiogenesis (Ozerdem et al. [2002], Ozerdem and Stallcup [2004]). NG2 has also been identified in some cancer cells, such as melanomas and glioblastomas (Stallcup [2002]). NG2 contributes to cell proliferation and motility. NG2 is an auxiliary receptor for growth factors such as Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor-AA (PDGF-AA) and integrins (You et al. [2013]). As with other proteoglycans, NG2 sequesters the factors presenting them to their receptors. NG2 also binds metalloprotease, which appears important in ECM degradation caused by tumour cells. Cell motility is probably enhanced by binding with collagen VI (Stallcup [2002]). NG2 has not been reported to be expressed by 10T1/2 cells.

6.1.4 Immunoglobulin of the class G (IgG) structure

The antibodies that will be used to functionalise the polymersomes are IgG. An IgG is a protein with a molecular weight of ≃150 Kda, and is the most prevalent isoform of antibody. It is composed of two identical long (heavy) chains (Molecular weight (MW)≃50 KDa) and two identical shorter (light) chains (MW≃25 KDa). Disulfide bonds link together the heavy chains and the light chains in the typical quaternary Y-shaped structure (see fig 6.4). There are also functional distinctions. The non-binding region (Fc fragment) is a highly conserved and glycosylated portion of the IgG. Ideally, any chemical modification should occur in this area as it is not involved in the antigen recognition. The two arms of the Y-shape (Fab fragment) includes the binding site. Knowing the exact amino acid composition and 3D structure of an IgG is important to identify the most probable binding site and the best binding strategy, to avoid compromising the antigen recognition efficiency.
Figure 6.3: (a) Affinity of the iso-form of PDGF for the PDGFR. Solid lines indicate high affinity, dotted lines indicate low affinity. (b) Reconstruction of the interaction of PDGF-BB dimer with the dimerised PDGFR-ββ. The two receptor monomers are represented in red and green and the two PDGF monomers are represented in blue and yellow. Protein Data Base (PDB) file obtained from Shim et al. [2010].
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Figure 6.4: IgG Structure. The two heavy chains are visible in red and blue, while the two light chains are coloured in green and yellow. Image obtained by visualisation of PDB file 1IGT (Harris et al. [1997]).

6.1.5 Conjugation methods

Nanoparticles can accumulate in tumours by taking advantage of increased leakiness and reduced lymphatic drainage (Enhanced Permeation and Retention (EPR), sec. 1.4.2). Side effects are limited by delivering the cargo preferentially to the tumour site, therefore increasing distribution volume and local concentration. Passive targeting can be coupled with active targeting to a specific cell population. Active targeting can be achieved by decorating the nanoparticle surface with specific antibodies to recognise cell markers.

There is a vast literature available on conjugation of molecules with differential composition (peptides, antibodies, nucleic acid, small molecules, dyes and more) to polymers. The process is called bio-conjugation and it has been developed mostly for polymer therapeutics. In 1975 Rigsdorf proposed a polymer-drug model characterised by a polymeric backbone conjugated to a drug and a targeting moiety (Ringsdorf [1975]). Over the years this model proved to efficiently improve the pharmacokinetics and pharmacodynamic profile of the drug by limiting side effects and improving drug efficacy due to the targeting moiety. Bio-conjugation has resulted in a number of Food and Drugs Administration (FDA) approved therapeutic agents, although mostly non actively targeted (Duncan et al. [2006]).

One of the simplest approaches is complexation. This has been widely used
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Amino acid Conjugation strategy

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Conjugation strategy</th>
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<tr>
<td>N-terminal</td>
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<tr>
<td>Glutamine</td>
<td>TGase mediated</td>
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<tr>
<td>Cysteine</td>
<td>Thiolation</td>
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<tr>
<td>Cysteine (2)</td>
<td>Oxidation, bridging (2 cysteine required)</td>
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<tr>
<td>Threonine</td>
<td>O-glycosilation</td>
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<tr>
<td>Lysine</td>
<td>Amino-functionalisation</td>
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<td>Asparagine</td>
<td>N-glycosilation</td>
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<td>Serine</td>
<td>O-glycosialtion</td>
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<tr>
<td>C-terminal</td>
<td>C-terminal functionalisation</td>
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Table 6.1: Sites of bioconjugation of proteins. Amino acid residuals offer a number of sites available for bioconjugation using different chemical strategies. Adapted from Pasut and Veronese [2012]).

to decorate polymers and liposomes with nucleic acids (Felnerova et al. [2004], Grinstaff et al. [1997], Li and Szoka Jr [2007], Kataoka et al. [2013]). It is not a direct chemical link and requires the interaction between anionic nucleic acids and cationic lipids or polymers. The use of cationic polymers is a major drawback of the system, due to rapid clearance and higher toxicity when compared to neutral polymers. Another popular strategy for non-covalent binding involves the use of biotin/streptavidin. Covalent binding offers a wide range of chemical strategies depending on the structure of the two molecules. When considering proteins, such as the IgG used in the present work, the main possibilities are listed in table 6.1. Once a conjugation strategy has been chosen, for instance targeting of primary amino groups with N-hydroxysuccinimide, it is helpful to assess the availability of leucin in the protein and the location with respect to the active site (see fig.6.5). Ideally the targeted amino group should be highly represented, but not easily available for reaction near the functional site of the protein, to avoid a potential reduction in target-recognition efficiency.
During polymersome functionalisation for targeting purposes, the targeting moiety is linked to the hydrophilic block of the co-polymer to direct it towards the exterior of the polymersome, where the target will be located. Functionalisation of nanoparticles can be performed before or self-assembly, as described for liposomes in the review published by Marques-Gallego (Marqués-Gallego and de Kroon [2014]). The considerations made for liposomes can be here extended to polymersomes. Pre self-assembly functionalisation involves the synthesis of the co-polymer-protein construct as a first step, followed by solubilisation of the reaction product with pristine co-polymer and polymersome self-assembly. In post self-assembly functionalisation, the pristine co-polymer is mixed with reactive co-polymer, polymersomes are prepared and finally the polymersomes are functionalised. Both techniques have advantages and disadvantages. In pre self-assembly functionalisation, a percentage of the functionalised co-polymer will be oriented towards the internal hydrophilic core, therefore it will not be useful for targeting purposes. However, post self-assembly functionalisation can be difficult due to of the steric hindrance generated by the packed hydrophilic brush. Furthermore, the non-fouling formulation will tend to repel proteins. Pre self-assembly functionalisation is not affected by these limitations, however the solvent and the reaction condition must not compromise protein stability (6.6).
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Figure 6.6: Polymersomes can be functionalised (A) before self-assembly or (B) after self-assembly. The choice of best strategy depends on the self-assembly conditions and ligand (IgG) stability.

Biotin/streptavidin conjugation  Biotin is a small (244.31 g · mol⁻¹) water soluble molecule. In bio-conjugation, more specifically biotinylation, the small size is one of the advantages since it does not affect the properties of the molecule to which it is linked. Biotinylated compounds bind strongly to avidin and streptavidin ($K_d \approx 10^{-14} \text{mol} \cdot \text{l}^{-1}$). Streptavidin and avidin have a similar secondary structure despite sharing only $\approx 30\%$ of the amino acid sequence. They are large tetramers ($\approx 53 \text{kg} \cdot \text{mol}^{-1}$) binding up to four biotin moieties. Affinity for biotin decreases at higher rate in avidin than in streptavidin after the first binding event. Furthermore, avidin is glycosylated and positively charged. Streptavidin/biotin functionalisation is used daily in laboratories, for instance in Immunohistochemistry (IHC) and Immunocytochemistry (ICC) to link biotinylated primary antibodies to dyes conjugated with avidin or streptavidin. Using this non-covalent binding strategy polymersomes have been functionalised to map their surface via Atomic Force Microscopy (AFM) (Battaglia et al. [2011]), to mimic leukocyte binding strategy through integrins or selectins (Hammer et al. [2008]) or to target macrophages in vitro (Brož et al. [2005]).

Maleimide conjugation  Maleimide is traditionally used to functionalise thiol groups via oxidation of the sulphur atom (Pasut and Veronese [2012]), typically cysteine. At low pH (6.5-7.5) it is a specific reaction, however at pH>8 it can undergo nucleophilic attack from primary amines (i.e. lysine), and this reaction is favoured over thiol functionalisation (6.7)(Brewer and Riehm [1967]).
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6.1.6 Aim

An interest application of the methodologies developed in the chapter 4 is the active targeting of perivascular cells with rhodamine labelled polymersomes for optical imaging in vivo. Targeting of perivascular cells is of interest because of their role in vessel stabilisation, particularly in the context of tumour angiogenesis. However, pericyte targeting is challenging because of the lack of specific extracellular markers. In this chapter the expression of two of the most commonly reported perivascular cells markers ex vivo (PDGFR-β and NG2) will be tested in an in vitro perivascular cells model embryonic cells 10T1/2. The expression in 10T1/2 will be compared to the expression in SVECs and MFC188, with the aim of identifying a marker expressed preferentially by the perivascular cells model, therefore suitable for active targeting. Once the marker is identified, two different conjugation strategies will be investigated in order to functionalise polymersomes to target the perivascular cells. Finally, the targeting efficiency of the conjugate will be tested in vitro.
6.2 Results

6.2.1 ICC

ICC was used to assess the expression level of NG2, PDGFR-β and α-SMA in 10T1/2, MFC188 and SVECs. The aim of this analysis was to confirm expression of perivascular cell markers on 10T1/2 cells and to identify a marker expressed by 10T1/2 and not SVECs or MFC188. This is critical for the purpose of functionalise polymersomes to selectively target perivascular cells. Transformation of 10T1/2 into perivascular cells was induced by exposure to TGF-β1, initially used at 1 ng/ml. The expression levels of NG2, PDGFR-β and α-SMA were assessed after 24h, 48h and 72h of treatment (fig. 6.8). Image analysis showed a small increase in expression of α-SMA after 48h compared to the 24h treatment. At the 72h time point there was no further increase (p>0.05). PDGFR-β expression was also increased after 48h compared to 24h, while at 72h the expression decreased compared to the control. NG2 ratio of expression at 24h was unchanged with respect to the control, similar to α-SMA and PDGFR-β. However, NG2 expression decreased further at 48h and 72h compared to the control, while the expression of PDGFR-β and α-SMA was increased (p> 0.05). The expression of the markers was affected by exposure time to TGF-β1, and this first result suggested an ideal treatment of 10T1/2 with 1ng/ml for 48h in order to maximise expression of PDGFR-β and α-SMA without losing the expression of NG2.

Figure 6.8: Quantification of expression of α-SMA, NG2 and PDGFR-β in 10T1/2 cells treated with 1ng/ml of TGF-β1 for 24h, 48h or 72h. ICC micrographs were analysed as reported in 3.3. Expression is compared to 10T1/2 cells not treated with TGF-β1 (*=statistical difference from untreated 10T1/2, U-test, p<0.05).
In a subsequent experiment the exposure to TGF-β1 was further optimised by assessing the expression of the same markers in 10T1/2 after treatment with a range of TGF-β1 concentration (1-10ng/ml). The exposure time was limited to 24h and 48h as it was observed previously that 72h had no significant positive effect on the expression of any of the markers (p>0.05). Results are reported in figures 6.12-6.14. α-SMA expression was higher after 48h treatment at every concentration used except 10ng/ml. At 24h exposure the highest expression was measured when 10ng/ml were used, although the increase was not statistically significant (p>0.05). At 48h a trend can be observed where the ratio of expression is on average decreasing with increasing TGF-β1 concentration, however there is no statistical significant difference between the different concentrations (p>0.05). PDGFR-β expression after 48h showed a remarked increase compared to the 24h treatment (p<0.05), with the only exception of 10ng/ml treatment as was observed for α-SMA. Another similarity to α-SMA expression is that at 24h the ratio is proportional with concentration, but in this case there was a decrease at 2ng/ml. At 48h the maximal expression was measured at 2ng/ml. NG2 expression showed a different pattern compared to α-SMA and PDGFR-β. Expression ratio was ≤1, with the exception of 2ng/ml and 5ng/ml at 24h treatment. At 1ng/ml for 48h showed a significant decrease in expression (p<0.05). Increased concentrations of TGF-β1 restored the ratio to ≃1 (no significance between treatment and control, p>0.05).

These results suggested that expression of perivascular markers in 10T1/2 in better induced by treatment with 2ng/ml of TGF-β1 for 48h. At this concentration and time the expression of both α-SMA and PDGFR-β was at the maximum observed, although the increase was not always significant, while the expression of NG2 was retained at a similar level compared to the control.
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Figure 6.9: Representative images for staining of $\alpha$-SMA for 10T1/2. (A, B) 10T1/2 cells. 10T1/2 cells were added with (B, G) 1ng/ml, (C, H) 2ng/ml, (D, I) 5ng/ml or (E, J) 10ng/ml of TGF-$\beta$. Cells were grown for (B, C, D, E) 24h or (F, G, H, I) 48h before imaging. Bar 18$\mu$m.
Figure 6.10: Representative images for staining of PDGFR-β for 10T1/2. (A, B) 10T1/2 cells. 10T1/2 cells were added with (B, G) 1ng/ml, (C, H) 2ng/ml, (D, I) 5ng/ml or (E, J) 10ng/ml of TGF-β1. Cells were grown for (B, C, D, E) 24h or (F, G, H, I) 48h before imaging. Bar 18µm.
Figure 6.11: Representative images for staining of NG2 for 10T1/2. (A, B) 10T1/2 cells. 10T1/2 cells were added with (B, G) 1ng/ml, (C, H) 2ng/ml, (D, I) 5ng/ml or (E, J) 10ng/ml of TGF-β1. Cells were grown for (B, C, D, E) 24h or (F, G, H, I) 48h before imaging. Bar 18µm.
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Figure 6.12: Quantification of expression of α-SMA in 10T1/2 cells treated with 1-10ng/ml of TGF-β1 for 24h or 48h. ICC micrographs were analysed as reported in 3.3. Expression is compared to 10T1/2 cells not treated with TGF-β1 (*=statistical difference from untreated 10T1/2, U-test, p<0.05. N=3, ±SD).

Figure 6.13: Quantification of expression of PDGFR-β in 10T1/2 cells treated with 1-10ng/ml of TGF-β1 for 24h or 48h. ICC micrographs were analysed as reported in 3.3. Expression is compared to 10T1/2 cells not treated with TGF-β1 (*=statistical difference from untreated 10T1/2 U-test, p<0.05. N=3, ±SD).
Additional experiments investigated the effect of growing condition on markers expression. The expression of \( \alpha \)-SMA because, despite being useful to confirm the perivascular nature of 10T1/2 cells, it cannot be used for targeting purposes as it is located inside the cell, therefore not accessible to polymersomes. In these experiments TGF-\( \beta \)1 was added at 2ng/ml for 48h. The effects of culturing 10T1/2 in conditioned media from MFC188 or SVECs and fibronectin coating of the well were assessed (fig. 6.17-6.16). In parallel to marker detection on 10T1/2, MFC188 and SVECs were probed for the same markers (fig. 6.18-6.16). Data quantification is reported in figure 6.19.

NG2 expression was significantly decreased in 10T1/2 cultured in MFC188 conditioned media (ratio 0.9, \( p < 0.05 \)) compared to untreated 10T1/2 cells, but there was no significant difference in any other culturing condition or in expression on MFC188 cells, when compared to 10T1/2 cultured in standard conditions (Basal medium eagle (BME), no TGF-\( \beta \)1 added). No expression of NG2 was detected in SVECs cells. PDGFR-\( \beta \) expression did not showed any statistical difference \( (p > 0.05) \) between 10T1/2 cultured in conditioned media and MFC188 or SVECs compared to 10T1/2 cultured in standard condition. High levels of expression of PDGFR-\( \beta \) was detected in NIH-3T3 cells, a positive control for the antibody used (Santa Cruz SC-432).
Figure 6.15: Representative images for staining of NG2 for 10T1/2. A) 10T1/2 cells, B) 10T1/2+2ng/ml TGF-β1, C) 10T1/2+2ng/ml TGF-β1 and fibronectin coated well, D) 10T1/2 cells in MFC188 conditioned media, E) 10T1/2 in MFC188 conditioned media and fibronectin coated well, F) 10T1/2 cells in SVECs conditioned media, G) 10T1/2 in SVECs conditioned media and fibronectin coated well. Bar 30µm.
Figure 6.16: Representative images for staining of NG2 for 10T1/2, MFC188 and SVECs. A) 10T1/2 cells, B) 10T1/2+2ng/ml TGF-β1, C) MFC188, D) SVECs. Bar 30µm.
Figure 6.17: Representative images for staining of PDGFR-β for 10T1/2. A) 10T1/2 cells, B) 10T1/2+2ng/ml TGF-β1, C) 10T1/2+2ng/ml TGF-β1 and fibronectin coated well, D) 10T1/2 cells in MFC188 conditioned media, E) 10T1/2 in MFC188 conditioned media and fibronectin coated well, F) 10T1/2 cells in SVECs conditioned media, G) 10T1/2 in SVECs conditioned media and fibronectin coated well. Bar 30µm.
Figure 6.18: Representative images for staining of PDGFR-β for 10T1/2, MFC188 and SVECs. A) 10T1/2 cells, B) 10T1/2+2ng/ml TGF-β1, C) MFC188, D) SVECs, E) 3T3 cells. Bar 30µm.
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Figure 6.19: Quantification of markers expression (NG2 and PDGFR-β) in 10T1/2 cultured in different conditions and in MFC188, SVECs and NIH/3T3. Expression was compared to 10T1/2 cells not treated with TGF-β1 (*=statistical difference from untreated 10T1/2, U-test, p<0.05. N=3, ±SD).

6.2.2 Western Blotting

Immunocytochemistry studies were complemented with western blot analysis for confirmation. Traditional western blot on acrylamide gel and quantitative western blot (ProteinSimple system) were used. In conventional western blot, NG2 expression in 10T1/2 treated for 48h with 2ng/ml of TGF-β1 is reduced by
50% when compared to the control, and no expression was detected in MFC188 or SVECs. Using the automated western blot equipment, different concentrations of TGF-β1 with 10T1/2 were evaluated, and a reduced NG2 expression in the presence of 5ng/ml and 10ng/ml (0.7 and 0.9 ratio) was observed, while with 2ng/ml there was a small increase in expression (1.05 ratio), although not significant (p>0.05). MFC188 and SVECs had very low expression (≃0.1). PDGFR-β detection was also different in the two methods. In traditional western blot there is a slight decrease in expression in 10T1/2 treated with 2ng/ml of TGF-β1 for 48h compared to the control (ratio 0.9). MFC188 has significant (p<0.05) reduced expression (ration 0.4) and no expression was detected in SVECs. Automated western blot detected an increase in PDGFR-β expression from all the 10T1/2 TGF-β1 treated cells, while the receptor was found to be expressed at significantly (p<0.05) lower levels in MFC188 and SVECs (ratio 0.35 and 0.15) (see fig. 6.20-6.21). These results suggested that both antibodies can be used to target the perivascular cells as they are expressed only (NG2) or at greater level (PDGFR-β) by 10T1/2 compared to SVECs or MFC188. Antibody against PDGFR-β will be the first one tested in polymersomes functionalisation using biotin/streptavidin chemistry.
Figure 6.20: Traditional western blot results for PDGFR-β and NG2. Expression was assessed in 10T1/2, 10T1/2 treated with TGF-β1 2ng/ml for 48h, MFC188 and SVECs.
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(a) Traditional western blot

(b) Quantitative automated western blot

Figure 6.21: Western blot results for the traditional method (top) on acrylamide gel and the quantitative automated method (bottom). All results expressed as ratio with respect of the band intensity or signal intensity measured in the control (10T1/2 cells not treated with TGF-β1) (*=statistical difference from untreated 10T1/2, U-test, p<0.05. N=3, ±SD).
Figure 6.22: Quantitative western blot chromatograms. A) PDGFR-β and B) NG2 detection. Expression was assessed in 10T1/2, 10T1/2 treated with increasing concentration of TGF-β1 (2-10 ng/ml) for 48h, MFC188 and SVECs. Expression is proportional to the area of the peak.

6.2.3 Biotin/streptavidin conjugation

To test the control over the conjugation ratio we conjugated different formulations obtained using different molar ratios (0, 0.5, 3 and 5%) of Poly(2-methacryloxyethyl-phosphorylcholine)-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA)-biotin with the same amount of streptavidin and biotiny-
lated Texas-Red (equivalent to 2/1 molar ratio with the highest concentration of PMPC-PDPA-biotin). The amount of dye linked to the polymersomes after purification was quantified (see figure 6.23). Results are expressed as fluorescence intensity normalised by the amount of PMPC-PDPA detected. It can be observed that when no PMPC-PDPA-biotin was used, no Texas-red was detected, and that the concentration of Texas-red increased linearly with the concentration of PMPC-PDPA-biotin detected, in the range 0.5%-5% molar.

![Figure 6.23: Polymersome biotinylation. Formulations containing 0%, 0.5%, 3% and 5% of PMPC-PDPA-biotin were reacted with streptavidin and Texas-Red-biotin (1:1 molar ratio with biotinylated co-polymer). After purification, samples were analysed by Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) and results are expressed by fluorescence intensity, proportional to Texas red concentration, normalised by the total amount of PMPC-PDPA detected in the UV channel at 220nm. N=3, ±SD.](image)

The antibody against PDGFR-β used in polymersome functionalisation was purchased biotinylated and it was different from the one used to evaluate marker expression. The use of this new antibody was validated using the positive control NIH-3T3 cells. In this experiment the biotinylated antibody to PDGFR-β was added to living NIH-3T3 cells and FITC-avidin was used to detect the biotinylated antibody. The average intensity was measured against a control, which was 3T3 cells treated with FITC-avidin, but not with the biotinylated antibody (figure 6.24). A clear increase in signal intensity was recorded, confirming that the antibody chosen is suitable to target living cells expressing PDGFR-β.
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Figure 6.24: Biotinylated antibody to PDGFR-β testing. Living NIH-3T3 cells were treated with biotinylated antibody to PDGFR-β (right) and FITC-avidin as a control (left). Bar 30µm.

**Polymersome functionalisation**

Biotinylated polymersomes were functionalised with antibody to PDGFR-β. The antibody was purchased already biotinylated and linked to the polymersomes via a streptavidin bridge. The reaction mixture was purified by Gel Permeation Chromatography (GPC) and characterised by TEM, Dynamic Light Scattering (DLS) and RP-HPLC, as described in sections 2.1.3-2.1.4. TEM and DLS data showed that spherical size and size distribution was not altered by the synthetic protocol. A slight increase in the peak of size distribution recorded by DLS (from ≈90nm to ≈110nm) suggesting successful conjugation which cause increase in hydrodynamic diameter of polymersomes (see fig. 6.25). RP-HPLC results indicated a molar ratio antibody/co-polymer of 2.1±1.2pmol/1mmol, demonstrating the presence of both co-polymer and antibody in the purified sample, therefore proving successful conjugation (see fig. 6.26).
Figure 6.25: (a, b) TEM and (c) DLS of polymersomes functionalised with biotin/streptavidin. Pristine polymersomes (red) are compared to 10% functionalised polymersomes (blue). Polymersomes retained spherical shape, however the hydrodynamic diameter was ≃20nm after conjugation, indicating surface modification.
Figure 6.26: *RP-HPLC* analysis for biotin/streptavidin functionalised polymersomes. Polymersomes peak at $\approx 7\text{min}$, the antibody peaks at $\approx 28\text{min}$, the purified conjugate polymersome-antibody showed both peaks ($\approx 7\text{min}$ an $\approx 28\text{min}$).

**Flow cytometry**

Uptake of functionalised formulations was evaluated by *Fluorescence Activated Cell Sorting* (FACS). The formulations tested with 10T1/2 cells were pristine Polyethylene Oxide-poly(2-(diisopropylamino)ethyl methacrylate) (PEO-PDPA), 1% functionalised PEO-PDPA, pristine PMPC-PDPA and 1% functionalised PMPC-PDPA. In this section of the work PEO-PDPA was used as a polymeric analogue of POEGMA-PDPA, which was not available in the laboratory at the time the experiments were performed. With MFC188 the pristine formulations of PEO-PDPA and PMPC-PDPA were tested. In 10T1/2 there was a small increase of functionalised PMPC-PDPA formulations uptake at 6h (+10%) and at 24h for functionalised PEO-PDPA formulations (+5%). However, the uptake was lower than that of the pristine formulations at the same time points from MFC188 (fig. 6.27).
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Figure 6.27: Uptake of functionalised polymersomes. Polymersomes were functionalised with 1% molar of antibody to PDGFR-β. Functionalised formulation did not show significant improvement in kinetic of uptake compared to the non-functionalised counterpart. N=3, ±SD.

6.2.4 Maleimide conjugation

Maleimide reaction was performed using a generic rabbit IgG instead of a specific antibody to PDGFR-β or NG2. This choice was justified considering the early developing stage of the functionalisation protocol and the consequent need for significant amount of material. A generic rabbit IgG represented a significantly cheaper alternative to specific antibodies. Maleimide reactivity can be shifted from thiol to primary amino groups by controlling the reaction pH (section 6.1.5) Considering the differences in amino acid abundance between cysteine and leucine in an IgG (fig. 6.5) the reaction was conducted at pH=7.5 (thiol groups, cysteine) and pH=8.5 (primary amino groups (leucine). TEM data showed spherical vesicles suggesting that the functionalisation protocol did not affect polymersomes stability. DLS data showed a small shift of size distribution from ≃90 nm to ≃100 nm, suggesting possible conjugation (fig. 6.28). RP-HPLC chromatograms were characterised by the presence of both peaks for polymer (fig. 6.29a) and the antibody (fig. 6.29b). However, the ratio between the two peaks was constant, indicating that there was no significant difference (p>0.05) between the two reaction yield and that the degree of functionalisation was the same (antibody/co-polymer 0.5±1.2 pmol/1 mmol).
Figure 6.28: (a, b, c) TEM and (d) DLS of polymersomes functionalised with maleimide. Pristine polymersomes (red) are compared to 10% functionalised polymersomes at pH 7.4 (blue) and pH 8.5 (black). Polymersomes retained spherical shape, however the hydrodynamic diameter was ~20nm after conjugation, indicating surface modification.
Figure 6.29: Maleimide reaction optimisation, RP-HPLC analysis. (a) Copolymer peaked at t ≃ 12.75 min. (b) IgG peaked at t ≃ 27.25 min. Representative RP-HPLC chromatograms for N=3 experiment. Peaks were integrated and the amount of IgG per polymersomes was calculated after normalising the area of the IgG against the area of the polymer.
6.3 Discussion

A fundamental requirement in targeted delivery is the identification of a specific marker selectively expressed in the cell to target. We choose to investigate the possibility to target perivascular cells. This choice was justified by the need of a better understanding the role of perivascular cells in angiogenesis and their response to anti-angiogenic or Vascular Disrupting Agent (VDA) treatments. Prof. Tozer’s group developed an in vivo tumour model of MFC188, and they characterised its perivascular cell coverage ex vivo. Polymersomes can allow direct, in vivo and real-time monitoring of perivascular cells in response to combretastatin. This is of great importance in order to assess the exact mechanism of response to the treatment and vasculature normalisation. Furthermore, it has potential beneficial ethical impact as it real-time observation is not an end-point procedure, therefore one animal can be used to acquired multiple time points, as opposed to ex vivo examination.

In the current chapter we investigated the expression of a number of proposed perivascular cells markers in 10T1/2 cells treated with TGF-β1, a cytokine reported to induce differentiation of perivascular cells (Hirschi et al. [1998]). Different concentrations and exposure times of TGF-β1 were tested, as well as different growing conditions. Furthermore we compared the expression of the markers in 10T1/2 cells, tumour and endothelial cells. This is of particular importance because the marker to be targeted is required to be expressed by the target cells, but not by other cell types such as tumour or endothelial cells; the aim of the study being to target perivascular cells in the tumour microenvironment.

The expression of three perivascular cells markers was assessed: α-SMA, PDGFR-β and NG2. α-SMA was included in the panel because it is characteristic of perivascular cells. However, it should be noted that it is located internally, therefore it cannot be used for targeting purposes. PDGFR-β and NG2 present extracellular epitopes that can be targeted (Shim et al. [2010], Stallcup [2002]).

Overall, ICC and Western Blot (WB) showed no significant effect of TGF-β1 or tissue culturing conditions on 10T1/2 expression of PDGFR-β, NG2 or α-SMA. It is also interesting to observe that some combinations of TGF-β1 exposure such as 1ng/ml for 48h or 72h caused an increased expression of α-SMA and decreased expression of NG2, which reflects a the phenotype of SMC surrounding veins (α-SMA+, NG2+/−) rather then capillary pericytes (α-SMA−, NG2+) (Armulik et al. [2011]).

Our results suggest that the pathway for differentiation of 10T1/2 in perivascular cells is likely to be dependent on other factors rather than only TGF-β1. Differentiation could be affected also by contact with endothelial cells (Hirschi
et al. [1998], Hirschi et al. [2003]), cell-signalling (TGF-β1-PDGF-B, 6.1.1), stiffness of the growth support (i.e. extracellular matrix, tubular shaped in vivo vs plastic well plate) unreported for 10T1/2, but well known for other stem cells systems (Park et al. [2011], Vincent et al. [2013]). Furthermore, it has been suggested that 10T1/2 might not fully differentiate into pericytes or vSMCs, but rather into myofibroblast with inconsistent markers expression (Brunelli et al. [2004], Yoshida and Owens [2005]). These conflicting reports have been explaining considering differences in protocols adopted by different laboratories (Xie et al. [2011]). Another possible explanation could be in the lack of a clear definition of pericytes and other perivascular cells, as most of the cited works referred to the differentiation of 10T1/2 in pericytes vSMCs, rather than specifically in pericytes.

A major limitation of the in vitro model is the lack of direct transferability to in vivo, as it is not possible to predict the expression of perivascular cells markers in the animal mode, as it would be inaccurate to assume that the endothelial cells of blood vessels found in the tumour growing in the Dorsal Skinfold Chamber (DSC) express the same markers as the SVECs used here. A better model to be adopted in future development of the targeting system would be to use histological samples from the animals. These samples have been already characterised for α-SMA and other markers expression by Prof. Tozer group, and targeting effect could be validated ex vivo using functionalised polymerosomes instead of the primary antibody normally used in histology.

The aim of this part of the work was not to elucidate the mechanism of differentiation of 10T1/2 into pericytes or vSMCs, but to assess if expression of perivascular cell markers by 10T1/2 was higher compared to the expression of the same marker in MFC188 or SVECs. With this respect, ICC showed no significant difference in expression of PDGFR-β or NG2 between the three cell types. WB, both the traditional acrylamide gel based and the quantitative Simon methods, showed significant reduced of expression of PDGFR-β and NG2 in MFC188 and SVECs compared to 10T1/2. The difference between the two techniques could be explained by a better sensitivity of WB method compared to ICC. Considering these results it was chosen to functionalise polymersomes with both both antibodies against PDGFR-β and NG2, however only the first one was used in preliminary in vitro screening, while a cheaper generic IgG was preferred as a model of antibody in further optimisation of the conjugation method.

In principle, the functionalisation can be achieved before or after polymersomes formation. When performed before formation, the single chain of copolymer is reacted with the desired moiety and the purified product is mixed with pristine copolymer in the second, separate step of polymersomes formation. This approach has the advantage of being easier to control and to facilitate
the reaction between the copolymer and the functionalising molecule. However, the reaction requires the co-polymers to not aggregate, and this often involves non-biocompatible chemical solvents that will need to be purified form the final product. Furthermore, when the functionalised copolymer is mixed with the pristine copolymer during polymersome formation, statistically, half the functionalised chains will be exposed to the inner aqueous core of the polymersomes rather than the outer surface. Such positioning is useless for functionalisation and will result in lowering the yield of reaction by about 50%. The second approach is to functionalise polymersomes after their formation. In this way only the reactive chains on the outer surface of the vesicles will react. This has the advantage of reducing the waste of functionalising reagent. However, such an approach presents increased steric hindrance from the polymersomes which could affect reaction yield. In this work, functionalisation was performed following polymersome self-assembly.

We tested two different reaction methods. The first reaction exploited the strong non-covalent binding between biotin and the multivalent streptavidin to link biotinylated copolymers with biotinylated antibody to PDGFR-β using a bridge of streptavidin. We showed that the conjugation can be controlled by changing the amount of biotinylated copolymer included in the polymersomes formulation, and that the antibody used is effective in recognising PDGFR-β. Facs analysis showed increase of uptake by 10T1/2 for both PEO-PDPA and PMPC-PDPA functionalised formulations compared to the pristine formulations. However, the increased uptake was still not significantly (p>0.05) higher compared to the pristine formulations by MFC188. This can be explained by low efficiency of functionalisation and because PDGFR-β is also expressed by MFC188.

Biotin/streptavidin polymersomes functionalisation required 3 steps: polymersomes self-assembly, streptavidin binding, biotinylated antibody binding. It will also involved several purification steps to eliminate intermediate unreacted reagents. These requirements have a negative impact on the cost of the production method and on the yield. To overcome these limitations we investigated the possibility using copolymers functionalised with maleimide to link directly thiol groups in the antibody (cysteine). This approach will allow to eliminate one reaction and purification step, and also to avoid the use of streptavidin, a vary large protein that could potentially interfere with polymersomes stability, mechanism of reaction or antibody/antigen recognition. The generic maleimide reaction is reported in figure 2.6. Considering the high cost of the reaction due to the amount of antibody required, the method was developed using a non specific rabbit IgG. Rp-Hplc analysis showed successful conjugation of the IgG, however at a very low yield (antibody/co-polymer ratio: 0.5±1.2pmol/1mmol).
Investigation of cysteine availability on IgG showed that the amino acid is poorly represented and generally not available, for instance because thiol groups are already engaged in S-S bridges. This could explain the low yield of reaction. Leucine is a more common amino acid and it has a reactive primary amino-group. Maleimide reactivity can be shifted to primary amino groups at higher (pH>8). The effect on reaction yield was tested by performing the reaction at pH=8.5. Results did not show improvement on the yield of reaction. This can be explained considering that Poly(2 - methacryloxyethyl - phosphorylcholine) (PMPC) is a non-fouling polymers, therefore it is possible that repulsive forces between polymersomes and IgG negatively affected the reaction kinetic. This can also explain the low reaction yield obtained from biotin/streptavidin approach. Further development of the should investigate the possibility of performing the reaction before polymersomes self-assembly.

Overall, we could not prove efficient in vitro targeting of perivascular cells. One of the major challenges associated with the project was the lack of a specific, highly expressed pericyte marker. 10T1/2 screening identified PDGFR-β and NG2 as possible markers, however they are not expressed only by 10T1/2, but also by tumour cells (MFC188), limiting their applicability in targeting. Furthermore, there is no proof that the marker will be expressed by tumour perivascular cells if the technology has to be translated to in vivo applications. Another problem was polymersomes-antibody conjugation strategy. When performed after polymersomes self-assembly, the reaction was characterised by low yield, most likely because of the steric hindrance generated by the non-fouling PMPC and Polyethylene Oxide (PEO) polymers.

Despite being negative for targeting purposes, the methods used identified the key steps in development of polymersomes for targeting: target identification, functionalisation and in vitro screening. The choice of alternative target (i.e. tumour cells via folic acid or RGD) and pre self-assembly conjugation can be tested in further development of the method.

A final consideration should be made on the choice of the targeting moiety. In this work we choose to work with a full IgG. This choice was made considering the commercial availability of biotinylated antibody to PDGFR-β and the possibility to optimise the conjugation with a generic rabbit IgG. However, to respect the stoichiometric ratio 1/1 between reactive co-polymer and antibody, it was necessary to use high mass of high molecular weight IgG (≃65KDa). Considering the cost of the biotinylated antibody, a single reaction with 10% reactive co-polymer can be estimated to require ≃40,000£ in consumable and reagents to obtain enough material for an in vitro uptake study. Furthermore, functionalisation with big molecules can compromise the stealth properties of polymersomes,
facilitating opsonisation and recognition by phagocytic cells. In future studies, 
IgG fragments (i.e. Fab) or smaller peptides should be considered instead of full 
IgG.
7.1 General discussion

In this work, polymersomes based on co-polymers Poly(2-methacryloxyethyl-phosphorylcholine)-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA) and poly(oligo(ethylene glycol) methacrylate)-poly(2-(diisopropylamino)ethyl methacrylate) (POEGMA-PDPA) have been used in conjunction with the Dorsal Skinfold Chamber (DSC) to image the tumour microenvironment. The development of vesicles started with the study of uptake in vitro. Live cell imaging was used as a quantitative method, its results being supported by Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) and Fluorescence Activated Cell Sorting (FACS) analysis (4.1). Because of the importance of blood flow in the dynamic of polymersomes recognition and internalisation by cells, the in vitro system was integrated with the development of a flow bio-incubator dedicated to quantification of uptake studies via FACS and live cell imaging (fig. 4.8). Uptake studies were performed on three cell types relevant to the tumour microenvironment: tumour cells (Mouse Fibrosarcoma Cells expressing VEGF188 isoform (MFC188)), endothelial cells (Small Vessel Endothelial Cells (SVECs)) and a perivascular cell model (10T1/2). Results showed a faster uptake of PMPC-PDPA polymersomes compared to POEGMA-PDPA by all cell types, and particularly by tumour cells (fig. 4.2). In flow conditions, the differences between all cell types and polymersome formulations were significantly reduced (fig. 4.11). Differences between PMPC-PDPA and POEGMA-PDPA could be surprising considering that both poly(oligo(ethylene glycol) methacrylate) (POEGMA) and Poly(2-methacryloxyethyl - phosphorylcholine) (PMPC) are not fouling, therefore they will not interact with circulating proteins which leads to opsonisation and rapid blood clearance. However, it has been reported that PMPC-PDPA polymersomes
uptake is mediated by scavenger receptor SR-B1 (Colley et al. [2014])). Reduced uptake in flow to a level similar to POEGMA-PDPA suggests that flow interferes with the recognition of PMPC-PDPA polymersomes by the receptors, therefore the uptake kinetic becomes similar to POEGMA-PDPA. Further development of the analysis in flow took advantage of the unique capability of imaging to focus in real time to different areas of the well. Because the device developed did not deliver perfect homogeneous flow at the cell level, the local differences were used to correlate shear stress and velocity magnitude to cellular uptake (fig. 4.12-4.14). Results suggested that shear stress had lower impact on cell uptake compared to vertical velocity. This suggests that the effect of flow on speed of the particles (therefore kinetics of ligand/receptor recognition) is higher than the effect of shear stress, which is likely to affect cell phenotype by inducing mechanical stress (Khan and Sefton [2011]), rather than ligand/receptor kinetics. Further studies are required to elucidate the effect of flow on distribution of SR-B1 at the cellular surface. Furthermore, uptake studies at this developmental stage were performed in cell media. This is a Newtonian fluid, very different to blood. It will be of interest to modify viscosity of the media to perform uptake studies in blood-like fluid. Overall, the in vitro findings suggested that PMPC-PDPA and POEGMA-PDPA formulations will have significantly different behaviour in vivo. PMPC-PDPA are expected to be cleared rapidly, while POEGMA-PDPA should have longer circulation time, which in turn will be reflected in improved accumulation within tumour.

In in vivo testing, the tumour vasculature was imaged in DSC using a confocal microscope equipped with a 10X objective. The tumour was implanted as a spheroid of MFC188. This particular model was chosen because it has been reported to be characterised by more vascularised tumours with a higher pericyte coverage compared to tumours obtained from Mouse Fibrosarcoma Cell (MFC) cells expressing Vascular Endothelial Growth Factor isoform 120 (VEGF120) or Vascular Endothelial Growth Factor isoform 164 (VEGF164) (Tozer et al. [2008], Akerman et al. [2013]). This tumour model was ideal to develop an imaging platform targeted to the tumour microenvironment. Image analysis was performed using a specifically developed algorithm. Both polymersome formulations were found to initially localise to the tumour vasculature, then gradually extravasate and accumulate within tumours. However, the analysis also confirmed the anticipated differences between PMPC-PDPA and POEGMA-PDPA polymersomes. POEGMA-PDPA accumulated within tumours to a greater extent compared to PMPC-PDPA polymersomes. After 16h from the injection, POEGMA-PDPA polymersomes were clearly localised in tumour stroma, with vessels visible in dark over the brighter stroma, while PMPC-PDPA polymersomes were hardly
detected in either the vessels or the stroma at the same time point (fig. 5.5, 5.6-5.6). This suggested faster clearance of PMPC-PDPA, which therefore was less efficient in accumulating within the tumour. These observation are in agreement with unpublished data from Prof. Battaglia showing longer circulating half life of POEGMA-PDPA compared to PMPC-PDPA polymersomes, which quickly accumulate in liver and spleen, organs primarily involved in nanoparticle clearance from blood. Finally, we implemented the analysis with a method to correlate the distance travelled by the polymersomes with the diameter of the source vessel. From the results it was observed that there was greater polymersomes accumulation around higher calibre vessels, however the furthest distance travelled is recorded from smaller vessels (fig. 5.8-5.9). The accumulation around bigger calibre vessels, and specifically near vessels branches, has been already reported for liposomes (Yuan et al. [1994]). A possible explanation could be that in those areas there is a higher local density of tumour macrophages up-taking and accumulating polymersomes. At smaller vessel level extravasated polymersomes are less likely to be sequestrated by phagocytic cells, therefore they can travel longer distances. This working hypothesis needs to be tested, ideally by macrophage labelling in vivo in the DSC followed by co-localisation study. Another possible explanation could be in the presence of bigger fenestrations in bigger calibre vessels, and local flow turbulence at the branching level facilitating polymersomes extrapolation.

The method of tumour targeting described is known as passive targeting. Polymersomes reach the tumour and accumulate within the stroma via Enhanced Permeation and Retention (EPR) effect. It is also possible to undertake active targeting to any specific tumour cell. Active targeting can be achieved by functionalising polymersomes with a moiety to recognise a specific cell marker. In the final part of the work an attempt at active targeting was introduced. The chosen target was perivascular cells. This cell type was chosen because of its key role in angiogenesis and vessels stabilisation, and the implication in development of pericyte targeted therapies. Targeting pericytes is challenging as there is no reported cellular marker specific of pericytes and uniquely expressed by pericytes. Furthermore, pericytes are not a well defined cell type. Restricting their definition to peri-capillary cells, there is still significant variation in reported markers depending on species, body location and activation status (Díaz-Flores et al. [2009]). For the in vitro work the embryo cell 10T1/2 was used a precursor of pericyte, as it has been reported that they can differentiate into perivascular cells in response to treatment with Transforming Growth Factor-β1 (TGF-β1) (Hirschi et al. [1998]). Adopting this model, the first part of the development was focused on assessing the expression of commonly accepted pericytes markers Platelet Derived Growth
Factor Receptor-β (PDGFR-β) and Nerve Glial antigen 2 (NG2) in 10T1/2 when treated with different concentrations of TGF-β1, and most importantly with respect to the expression of the same markers in MFC188 and SVECs. Ideally, the marker should be expressed only by 10T1/2 to facilitate active targeting. Results indicated that 10T1/2 expression of PDGFR-β and NG2 was not significantly affected by treatment with TGF-β1 alone, suggesting a more complex activation pathway, perhaps involving direct contact with endothelial cells in a co-culture system, as suggested by results reported by D’Amore and Hirschi (Hirschi et al. [1998], Hirschi et al. [1999]). However, expression of both markers, and particularly NG2, was found to be significantly higher in 10T1/2 compared to MFC188 and SVECs (fig. 6.12-6.21).

There is a vast library of chemistry available for polymer functionalisation. In this work we tested two different synthetic approaches that can be performed after polymersomes formation. The first approach is based on biotin/streptavidin chemistry, the second on maleimide reaction. In biotin/streptavidin approach a percentage of biotinylated co-polymer was included in the formulation and after polymersomes self assembly a biotinylated antibody against PDGFR-β was linked via a bridge of streptavidin. Maleimide is commonly used to functionalise thiol groups (cysteine in proteins), however at slightly higher pH it can also undergo nucleophilic attack from primary amino groups (leucine). Both strategies were tested. RP-HPLC characterisation (fig. 6.26, 6.29) showed successful conjugation for both strategies. Biotin/streptavidin conjugate was tested in vitro (fig. 6.27). At 6h a slight increased uptake of PMPC-PDPA functionalised formulation was observed in 10T1/2 cells, however the uptake was still lower compared to uptake of pristine PMPC-PDPA by MFC188. A similar pattern was observed for Polyethylen Oxide-poly(2-(diisopropylamino)ethyl methacrylate) (PEO-PDPA) functionalised polymersomes at the 24h time point.

Overall, results obtained in this work represent a collection of methods developed to screen polymersomes uptake in vitro, also taking into account the complexity of flow mediated particles delivery. Furthermore, dedicated in vivo analytical methods were developed to characterise accumulation of polymersomes within tumours. Results suggested that longer circulating POEGMA-PDPA polymersomes have better chance to accumulate intra-tumour and to provide increased signal-to-noise ratio compared to PMPC-PDPA. Furthermore POEGMA-PDPA are less likely to be up-taken preferentially by tumour cells by scavenger receptors. However, PMPC-PDPA can still be considered especially for short term imaging and perhaps active targeting of cells located close to the vessels, where no significant intra-tumour accumulation is required.
7.2 Future directions

The work presented generated some interesting questions that can be addressed in future developments.

**In flow** studies suggested a strong effect of flow on cellular uptake, and perhaps a more significant effect of velocity magnitude compared to shear stress. Further studies could complement the observations made by correlating uptake with topological cellular distribution of receptor involved in uptake pathways such as scavenger receptors. This is critical especially for endothelial cells, which are known to polarise receptors expression in relation to the vascular or avascular side. Formulations with receptor mediated uptake such as PMPC-PDPA could be affected by a polarisation of the receptor expression. Furthermore, it would be interesting to analyse polymersomes uptake not only as a function of the shear stress or the fluid velocity but also according to local polymersomes velocity or concentration, both quantifiable with appropriate *in silico* simulation. Polymersomes velocity is likely to be strongly correlated to fluid velocity, and to be very low at the boundaries (i.e. cell surface). Local polymersomes at the cellular level, however, is likely to be significantly higher at the inlet and outlet level compared to the rest of the well. This is because the inlet and outlet are two small points were all particles are forced to pass, while they can spread across a much bigger surface in the rest of the well. Furthermore, polymersomes concentration at the cellular level is probably very low because they are mostly quickly dragged away from the cell surface by the fluid, which has greater velocity away from the boundaries. If *in silico* simulations shows a significant effect on polymersomes drag, the device should probably be re-designed in favour of a greater surface to volume ratio compared to the current design. Finally, the introduction of a more viscous, blood-like fluid will improve the reproduction of physiological capillary flow.

**Active targeting** attempt was not successful. Targeting of perivascular cell is made difficult by the lack of a well characterised marker and the difficulty of translating *in vitro* models to *in vivo* models. Further work could introduce the screening on *ex vivo* samples obtained from animal models bearing the same MFC188 tumour model used in this work. More work can also be done in the conjugation of a targeting moiety to the polymersomes surface. In this work we choose to functionalise pre-formed polymersomes, however the formulations used are non-fouling, and this is likely to reduce the reaction yield as they will interact poorly with the **Immunoglobulin of the class G (IgG)** chosen as function-
alising molecule. Performing the reaction before the self-assembly could improve the reaction yield by reducing the steric hindrance. Furthermore, the use of a full antibody is not advisable for both stability and synthetic costs reasons. A full antibody has a relatively large hydrodynamic radius which could potentially destabilise the polymersome construct. Full antibodies could also be more easily recognised by the Reticuloendothelial System (RES) or opsonising proteins because of their large volume. Finally, the use of specific, full antibodies is costly, as it is necessary to use a significant amount of molecules, therefore of antibody, to perform the reaction in the ideal condition of antibody excess. The use of smaller molecules such as peptides or antibody fragments could address all the discussed issues.

Finally, in vivo image analysis has proved to be potentially useful in analyse a number of parameters related with polymersomes extravasation and intra-tumour accumulation. More work can be done to fully automate the masking process, which at the moment relies on the user judgement at least on the choice of the masking parameters such as intensity threshold or Difference of gaussians (DoG) kernel size. More animals are also required to draw significant conclusions from the analysis, as only a total of 5 animals divided across different formulations and imaging techniques were used. With respect to this, the surgical procedure necessary to implant the DSC proved to have poor outcome, with major complications arising a few hours after surgery. Some of the complication have been addressed already by changing some of the parts of the chamber, most importantly the spacers between the two frames. However, more work should be done to assure more reliable outcome, which has implication on both the ethics of the work and data quality from in vivo experimentation.
# Chapter 8

## List of Material Used

### 8.1 General *in vitro* laboratory equipment and reagents

<table>
<thead>
<tr>
<th>Consumables</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2ml cryogenic vials</td>
<td>Nalge Company, Rochester, NY, USA</td>
</tr>
<tr>
<td>0.5ml, 2.0ml eppendorf tubes</td>
<td>Starstedt, Ak &amp; Co, Germany</td>
</tr>
<tr>
<td>25cm², 75cm² tissue culture flasks</td>
<td>Nunc-Life Technologies Ltd, UK</td>
</tr>
<tr>
<td>3M autoclave tape</td>
<td>Rexam Medical Packaging, UK</td>
</tr>
<tr>
<td>5ml, 10ml and 25ml pipettes</td>
<td>Corning Incorporated, USA</td>
</tr>
<tr>
<td>70% (v/v) IMS</td>
<td>Adams Healthcare Ecolab, UK</td>
</tr>
<tr>
<td>Trigene</td>
<td>Medimark Scientific, UK</td>
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<tr>
<td>96, 24 well plates</td>
<td>Nunc-Life Technologies Ltd, UK</td>
</tr>
<tr>
<td>96 wells imaging plates</td>
<td>Becton, Dickinson and Company, NJ, USA</td>
</tr>
<tr>
<td>24 wells imaging plates</td>
<td>ibidi, Martinsried, Germany</td>
</tr>
<tr>
<td>Gilson pipette tips</td>
<td>Starsted Laboratory Supplies Ltd, UK</td>
</tr>
<tr>
<td>Latex examination gloves</td>
<td>Kimberly Clarke Ltd, UK</td>
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<tr>
<td>P2, P10, P20, P200, P1000 pipettes</td>
<td>Gilson Inc, USA</td>
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<tr>
<td>Paper tissue</td>
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<td>Presept tablets</td>
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<td>Sterile universal tubes</td>
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<td>Bijoux</td>
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<tr>
<td>Glass vials</td>
<td>Scientific Laboratory Supplies Ltd, UK</td>
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</table>
8. List of material used

8.1. GENERAL IN VITRO LABORATORY EQUIPMENT AND REAGENTS

1ml, 2ml, 5ml syringes  Becton Dickinson Ltd, UK
1ml insulin syringe  Becton Dickinson Ltd, UK
25G, 27G and 29G needles  Becton Dickinson Ltd
4 well slides  Nunc-Life Technologies Ltd, UK
Microscope glass slides  Fisher Scientific, UK
Coverslip  Fisher Scientific, UK
PVDF membrane  EMD Millipore Corporation, Billerica, MA, USA
Photographic film  GE Healthcare, UK
Marprene tubing  Watson-Marlow Pumps Group, UK
Elbow Luer Connector  ibidi, Martinsried, Germany
Carbon coated TEM Grids  Elektron Technology Ltd, UK
Cell scraper  Corning, NY, USA
Simon ProteinSimple consumables  ProteinSimple, Santa Clara, CA, USA

Table 8.1: In vitro consumables.

**Equipment**

**Item**  
5% CO₂ incubator  Sanyo, UK
Autoclave  Prestige Medical Ltd, UK
Centrifuge (tissue culture)  Eppendorf 5702R
Microcentrifuge  Eppendorf 5425
Ice machine  Scotsman Ice System, UK
pH meter  Hanna Instruments, Rhode Island, USA

Plate reader  
Fluorescence microscope Pathway 855  Microscope: Becton Dickinson Ltd, UK
Camera: Hammamatsu

Fluorescence microscope Leica TCS SP8  Microscope: Leica, Wetzlar, Germany
Camera: CRI NuanceFX, Quorum Technologies, CA, USA

Brightfield microscope  Medline Scientific Ltd, UK
Spectrofluorometer  Jasco Inc, USA
Sterile safety cabinets/hoods  Walker Safety Cabinets Ltd, UK
Temperature control water bath  Fisher Scientific, UK
Vortex  Whirlimixer, Fisher Scientific, UK
Analytical weighting scale  Sartorius, D
Technical weighting scale  Sartorius, D
Western blot equipment

Western Blot semi-dry membrane transfer

FACS CellArray

TEM microscope

Glow discharger

Peristaltic pump

Simon ProteinSimple

RP-HPLC Ultimate 3000

RP-HPLC column

Bio-Rad Laboratories, CA, UK

Bio-Rad Laboratories, CA, UK

Becton Dickinson Ltd, UK

Microscope: Fei Tecnica Spirit

Camera: Gatan1K MS600CW CCD

Quorum Technologies Ltd, UK

Watson-Marlow Pumps Group, UK

ProteinSimple, Santa Clara, CA, USA

Thermo Scientific Dionex, Waltham, Massachusetts, USA

Phenomenex, CA, USA

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Reagents

Table 8.2: *In vitro* equipment.

<table>
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<th>Item</th>
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<td>Hydrochloric acid</td>
<td>Sigma-Adrich, MI, USA</td>
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<tr>
<td>Sodium hydroxide</td>
<td>Sigma-Adrich, MI, USA</td>
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<td>BOC, UK</td>
</tr>
<tr>
<td>Gaseous Nitrogen</td>
<td>BOC, UK</td>
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<tr>
<td>Phosphotungstenic acid</td>
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<tr>
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<td>Lonza, Switzerland</td>
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<tr>
<td>BME</td>
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<td>Penicillin/streptomycin</td>
<td>Sigma-Adrich, MI, USA</td>
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<tr>
<td>Fungizone</td>
<td>Life Technologies, CA, USA</td>
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<tr>
<td>Methanol</td>
<td>Sigma-Adrich, MI, USA</td>
</tr>
<tr>
<td>Choloroform</td>
<td>Sigma-Adrich, MI, USA</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>Sigma-Adrich, MI, USA</td>
</tr>
<tr>
<td>PBS</td>
<td>Fisher Scientific, UK</td>
</tr>
<tr>
<td>FCS</td>
<td>Biosera, UK</td>
</tr>
<tr>
<td>Trypsin/EDTA</td>
<td>Sigma-Adrich, MI, USA</td>
</tr>
<tr>
<td>FACS cleaning buffer</td>
<td>Becton Dickinson Ltd, UK</td>
</tr>
<tr>
<td>FACS running buffer</td>
<td>Becton Dickinson Ltd, UK</td>
</tr>
<tr>
<td>RIPA Buffer</td>
<td>Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma-Adrich, MI, USA</td>
</tr>
<tr>
<td>Photographic fixative</td>
<td>Eastman Kodak Company, NY, USA</td>
</tr>
<tr>
<td>Photographic developer</td>
<td>Eastman Kodak Company, NY, USA</td>
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</table>
8. List of material used

8.1. General in vitro laboratory equipment and reagents

Simon ProteinSimple reagents  
ProteinSimple, Santa Clara, CA, USA

Fibronectin  
Sigma-Adrich, MI, USA

HEPES buffer  
Sigma-Adrich, MI, USA

Acetone  
Sigma-Adrich, MI, USA

Ethanol  
Sigma-Adrich, MI, USA

ABC  
Vector Laboratories, USA

DAB  
Vector Laboratories, USA

DPX  
Sigma-Adrich, MI, USA

Haematoxylin  
Sigma-Adrich, MI, USA

Xylene  
Sigma-Adrich, MI, USA

Hydrogen peroxide  
Sigma-Adrich, MI, USA

Rabbit IgG  
Sigma-Adrich, MI, USA

Texas red-avidin  
Invitrogen, CA, USA

FITC-avidin  
Invitrogen, CA, USA

Streptavidin  
Vector Laboratories, USA

Normal Horse Serum  
Vector Laboratories, USA

DAPI  
Vector Laboratories, USA

TGF-β1  
R&D Systems Inc, USA

Table 8.3: In vitro reagents.

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<thead>
<tr>
<th>Antibody</th>
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<tbody>
<tr>
<td>RGS5</td>
<td>sc-28492</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Desmin</td>
<td>ab15200</td>
<td>Abcam</td>
</tr>
<tr>
<td>α-SMA</td>
<td>A2547</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>sc-432</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>PDGFR-β-Biotin</td>
<td>ab93533</td>
<td>Abcam</td>
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<tr>
<td>NG2</td>
<td>ab5320</td>
<td>Millipore</td>
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<td>NG2</td>
<td>4235</td>
<td>Cell Signaling</td>
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<td>NG2-Alexa488</td>
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<td>CD31-FITC</td>
<td>102406</td>
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<tr>
<td>DyLight-649</td>
<td>406406</td>
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<tr>
<td>DyLight-488</td>
<td>406404</td>
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Table 8.4: List of all the antibodies used in vitro.

Consumables

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<tbody>
<tr>
<td>Syringes</td>
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</tr>
<tr>
<td>2.0ml eppendorf tubes</td>
<td>Sarsted AG &amp; Co, Germany</td>
</tr>
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8. List of material used

8.1. GENERAL IN VITRO LABORATORY EQUIPMENT AND REAGENTS

<table>
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<tr>
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<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>25G and 27G needles</td>
<td>Becton Dickinson Ltd, UK</td>
</tr>
<tr>
<td>4/0 Ethilon</td>
<td>Ethicon Ltd, UK</td>
</tr>
<tr>
<td>5/0 mersilk non-adsorbable suture</td>
<td>Ethicon Ltd, UK</td>
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<tr>
<td>5/0 PDS II suture</td>
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<tr>
<td>Latex examination gloves</td>
<td>Kimberly Clarke Ltd, UK</td>
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<tr>
<td>Microscope slides</td>
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<tr>
<td>Paper tissue</td>
<td>Kimberly Clarke Ltd, UK</td>
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<tr>
<td>Petri dish</td>
<td>Corning Inc, UK</td>
</tr>
<tr>
<td>Size 15 scalpel blade</td>
<td>Swann Morton, UK</td>
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<tr>
<td>Sterile Biogel gloves</td>
<td>Regent Medical Ltd, UK</td>
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<tr>
<td>Cotton swabs</td>
<td>Shermond Surgical Supply Ltd, UK</td>
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Table 8.5: *In vivo* consumables.

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<td>C-clamp</td>
<td>Medical Workshop, University of Sheffield, UK</td>
</tr>
<tr>
<td>Chamber pieces</td>
<td>Medical Workshop, University of Sheffield, UK</td>
</tr>
<tr>
<td>Confocal Microscope</td>
<td>Zeiss LSM510 NLO Upright</td>
</tr>
<tr>
<td>Epiluorescence Microscope</td>
<td>Nikon Eclipse E600FN</td>
</tr>
<tr>
<td>Restrainer (model 84)</td>
<td>IITC Life Science, USA</td>
</tr>
<tr>
<td>Incubator</td>
<td>Brinsea Products Ltd, UK</td>
</tr>
<tr>
<td>Sensitive warming pad</td>
<td>Cole-Palmer Ltd, UK</td>
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<tr>
<td>Surgical instruments</td>
<td>Fine Science Tools, USA</td>
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Table 8.6: IVM equipment.

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<th>Supplier</th>
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<tr>
<td>70% IMS</td>
<td>Adams Healthcare Ecolab, UK</td>
</tr>
<tr>
<td>Betadine surgical scrub</td>
<td>Seton Healthcare Group plc, UK</td>
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<tr>
<td>Trigene disinfectant concentrate</td>
<td>Medichem International, UK</td>
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Table 8.7: *In vivo* reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
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<tbody>
<tr>
<td>Tris-HCl</td>
<td>50mM</td>
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Lysis buffer
8. List of material used
8.1. GENERAL IN VITRO LABORATORY EQUIPMENT AND REAGENTS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
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<tbody>
<tr>
<td>NaCl(150mM)</td>
<td>150mM</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.02% (w/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Igepal CA-630 or Nonidet P-40</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>0.5%</td>
</tr>
<tr>
<td>Protease inhibitor</td>
<td>1 tablet for 10ml of buffer</td>
</tr>
<tr>
<td>Final pH</td>
<td>8.8</td>
</tr>
</tbody>
</table>

**Running buffer 10X**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
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</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>25mM</td>
</tr>
<tr>
<td>Glycin</td>
<td>0.192M</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1% (w/v)</td>
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<tr>
<td>Final pH</td>
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</table>

**Transfer buffer**

<table>
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</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>3.03g</td>
</tr>
<tr>
<td>Glycin</td>
<td>14.10g</td>
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<tr>
<td>SDS</td>
<td>2.5g</td>
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<tr>
<td>dH2O</td>
<td>900ml</td>
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<tr>
<td>Methanol</td>
<td>100ml</td>
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<tr>
<td>Final pH</td>
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<tr>
<td>SDS</td>
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</table>

**Stacking gel**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
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<tr>
<td>dH2O</td>
<td>3ml</td>
</tr>
<tr>
<td>tris-HCl pH 8.8</td>
<td>1.25ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>650µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5µl</td>
</tr>
</tbody>
</table>

**Running gel**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>4.85ml</td>
</tr>
<tr>
<td>tris-HCl pH 8.8</td>
<td>2.5ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5µl</td>
</tr>
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Table 8.8: Western blot reagents composition.
<table>
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<tr>
<th>Formulation as referred to in the text</th>
<th>Copolymer</th>
<th>Amount [mg]</th>
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<tr>
<td>PMPC-PDPA</td>
<td>PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Rhd-PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>2</td>
</tr>
<tr>
<td>PEO-PDPA</td>
<td>PEO\textsubscript{113}-PDPA\textsubscript{70}</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Rhd-PEO\textsubscript{113}-PDPA\textsubscript{70}</td>
<td>2</td>
</tr>
<tr>
<td>POEGMA-PDPA</td>
<td>POEGMA-PDPA</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Rhd-POEGMA-PDPA</td>
<td>2</td>
</tr>
<tr>
<td>Biotin-PMPC-PDPA</td>
<td>PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Rhd-PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Biotin-PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>2</td>
</tr>
<tr>
<td>Maleimide-PMPC-PDPA</td>
<td>PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Rhd-PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Maleimide-PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>2</td>
</tr>
<tr>
<td>Maleimide-POEGMA-PDPA</td>
<td>POEGMA\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Rhd-POEGMA-PDPA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Maleimide-PMPC\textsubscript{25}-PDPA\textsubscript{70}</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 8.9: Composition of the different polymersomes formulations use. The amounts reported are intended for the reparation of 2ml of polymeromses in film hydration or to be dissolved in 2ml of Phosphate Buffer Saline pH 2 (PBS\textsubscript{2}) using pH switch technique.
9.1 Binding efficiency (BE)

Binding efficiency (BE) was defined as the amount of polymersomes up-taken by the cells in the unit of time. BE was calculated by combining data from Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) (amount of polymersomes) and Fluorescence Activated Cell Sorting (FACS) (sorting of cell population positive for polymersomes) analysis. The work flow for the analysis is reported in figure 9.1.
Figure 9.1: Binding efficiency calculation. FACS results were plotted in logarithmic scale and the time-point at which the plateau of maximal uptake was reached ($t_p$) was graphically extrapolated. The number of vesicles per cell internalised was derived from RP-HPLC data at $t = t_p$.

RP-HPLC and FACS analysis were performed comparing 10% rhodamine labelled Polyethylen Oxide-poly(2-(diisopropylamino)ethyl methacrylate) (PEO-PDPA) and Poly(2 - methacryloxyethyl-phosphorylcholine)-poly( 2 -(diisopropylamino)ethyl methacrylate) (PMPC-PDPA) polymersomes. Cells used were Mouse Fibrosarcoma Cells expressing VEGF120 isoform (MFC120), Mouse Fibrosarcoma Cells expressing VEGF188 isoform (MFC188) and 10T1/2. Results are reported in figure 9.2-9.3. Consistently with the reported profile of uptake described in section 4, PMPC-PDPA polymersomes were up-taken faster than PEO-PDPA polymersomes. It was also confirmed higher uptake from Mouse Fibrosarcoma Cell (MFC) compared to 10T1/2. There was no significant difference between MFC120 and MFC188 ($p>0.05$).
9.1. BINDING EFFICIENCY (BE)

Figure 9.2: RP-HPLC uptake results. PMPC-PDPA (solid lines) is up-takes faster than PEO-PDPA, with no significant difference \((p>0.05)\) between MFC188 (red lines) and MFC120 (black lines). 10T1/2 (blue lines) have very low uptake of PEO-PDPA, and the uptake of PMPC-PDPA is higher than PEO-PDPA, however it is lower than the uptake of the same formulation in MFC cell lines. \(N=3, \pm SD\).

Figure 9.3: FACS uptake results. PMPC-PDPA (solid lines) is up-takes faster than PEO-PDPA, with no significant difference \((p>0.05)\) between MFC188 (red lines), MFC120 (black lines) and 10T1/2 (blue lines). Uptake of PEO-PDPA has no significant difference \((p>0.05)\) between the three cell lines up to 6h, however at 24h there is a significant difference \((p<0.05)\) between MFC cell lines (higher uptake) and 10T1/2. \(N=3, \pm SD\).

To calculate BE, it was critical to reach a plateau of uptake. with PEO-PDPA
this was not achieved in the time limit of the analysis. The value of maximal uptake can be mathematically extrapolated, however this would introduce inaccuracy. For this reason the calculation of BE was limited to the two MFC cell types. BE of PMPC-PDPA was significantly higher (p > 0.05) than BE of PEO-PDPA polymersomes, and there was no significant difference (p > 0.05) between MFC120 and MFC188 (9.4). The method provided a useful absolute term of comparison between uptake of different formulations form different cell types. However the technique was not developed further as it required to perform both FACS and RP-HPLC for any given combination of polymersome formulation and cell type. This was not advisable in fast, high throughput screening.

Figure 9.4: Binding efficiency for PEO-PDPA and PMPC-PDPA in MFC188 and MFC120. PMPC-PDPA had greater BE compared to PEO-PDPA, while there was no significant difference between MFC120 and MFC188. N=3, ±SD.

9.2 FACS for in flow uptake

In section 4 FACS results were reported as fluorescence intensity variation over time. This was useful to compare imaging and FACS results. However, FACS results can also be expressed as variation of percentage of cells positive for polymersomes over time. In this case there is no information on magnitude of uptake, but only on the percentage of cells that are up-taking polymersomes at a higher rate than a fixed threshold. For this reasons the two plots can be different, as it is possible that all cells are up-taking polymersomes, therefore the percentage of population is high, however the uptake could be slow, resulting in low magnitude of uptake. This can be observed in the plots here reported (9.5-9.7) when compared to plots 4.11, especially for cell types with low uptake (Small Vessel Endothelial Cells (SVECs) and 10T1/2). Furthermore, it can be observed how FACS uptake curves expressed as percentage of population reached a plateau
for PMPC-PDPA and MFC188 and SVECs, but no plateau was observed in curves expressed as variation of intensity. This suggested that for fast uptake polymersomes all cells became positive for polymersomes, and vesicles continued to accumulate within the cells increasing the intensity of the fluorescence signal.

Figure 9.5: Uptake of PMPC-PDPA and poly(oligo(ethylene glycol) methacrylate)-poly( 2 -((diisopropylamino)ethyl methacrylate) (POEGMA-PDPA) rhodamine labelled polymersomes by MFC188. FACS data expressed as percentage of cells positive for rhodamine labelled polymersomes. N=3, ±SD.

Figure 9.6: Uptake of PMPC-PDPA and POEGMA-PDPA rhodamine labelled polymersomes by SVECs. FACS data expressed as percentage of cells positive for rhodamine labelled polymersomes. N=3, ±SD.
9.3 In vivo epifluorescence imaging

The epifluorescence microscope routinely used to assess tumour establishment in Dorsal Skinfold Chamber (DSC) was also used to acquire fluorescence image of injected polymersomes and for image analysis. Representative images acquired equipping the microscope with a 2.5X objective and a 20X objective are reported in figure 9.8. Images were acquired 5 min after the injection of rhodamine labelled POEGMA-PDPA polymersomes. In 2.5X and 20X transmitted light images (9.8-a,c) vessels can be observed in dark over brighter stroma, however in 20X images the contrast was significantly reduced. As was observed in confocal images (5.3), at 5 min after injection vessel in rhodamine channel are highlighted in bright over dark background.

Figure 9.7: Uptake of PMPC-PDPA and POEGMA-PDPA rhodamine labelled polymersomes by 10T1/2. FACS data expressed as percentage of cells positive for rhodamine labelled polymersomes. N=3, ±SD.
Figure 9.8: DSC images obtained with different magnification, epifluorescence microscopy. All images were acquired 5min after Intra Venous (i.v.) injection of rhodamine labelled POEGMA-PDPA polymersomes. On the left (a, c) transmitted light pictures, on the right (b, d) fluorescence pictures. Images (a, b) were acquired with a epifluorescence microscope equipped with a 2.5X objective, images (c, d) using the same microscope equipped with a 20X objective. Note how, in the rhodamine channel, vessels appeared bright over darker tumour stroma. Bar(a, b) 710µm; (c, d) 90µm.

A time lapse of both formulations is reported in figure 9.9-9.10 for 2.5X and 20X magnification respectively. In both cases it was observed that at early time points vessels were highlighted by polymersomes in the rhodamine channel, however at later time points vessels appeared in dark over brighter background. This supported confocal analysis results and suggested polymersomes extravasation and accumulation within tumour via Enhanced Permeation and Retention (EPR) effect.
Figure 9.9: MFC188 tumour microvasculature imaged in epifluorescence, 2.5X objective. Note how between 2min and 30min after injection the tumour vasculature gradually became brighter and the contrast increased, while at 15h vessels appeared dark over brighter stroma. Tumour region of the image was manually masked using the image analysis software developed (sec. 3.6). Bar 710µm.
Figure 9.10: *MFC188* tumour microvasculature imaged in epifluorescence, 20X objective. Note how between 2 min and 30 min after injection the tumour vasculature gradually became brighter and the contrast increased, while at 15 h vessels appeared dark over brighter stroma. Tumour region of the image was manually masked using the image analysis software developed (sec. 3.6). Bar 90 µm.

Images were analysed using the algorithm described in section 3.6 to assess polymersomes intra-tumour distribution. Results for both 2.5X and 20X magnification confirmed what observed in confocal microscopy: at early time points the vessels have higher contribute to fluorescence signal strength compared to tumour stroma, while at later time point the relative contribution was reversed. However,
epifluorescence imaging was affected by out-of-focus light, limiting sensitivity and accuracy of the analysis (see 5.6-5.7 compared to 9.11-9.12).

Figure 9.11: **POEGMA-PDPA** in vivo image analysis. Data are represented as overall mean intensity of rhodamine signal in the tumour area (right axis) overlaid with a break down of the relative contribution of vessels, edges and tumour stroma to the signal intensity (left axis, coloured bars). Epifluorescence microscopy, 2.5X objective. N=2.
Figure 9.12: **POEGMA-PDPA** in vivo image analysis. Data are represented as overall mean intensity of rhodamine signal in the tumour area (right axis) overlaid with a break down of the relative contribution of vessels, edges and tumour stroma to the signal intensity (left axis, coloured bars). Epifluorescence microscopy, 20X objective. \(N=2\).
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<td>Causes of death in 2011 and cancer incidence in 2010</td>
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<td>1.2</td>
<td>Properties of the vascular system with respect to size</td>
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<td>1.3</td>
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<td>Typical Transmission Electron Microscopy (TEM) results</td>
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<td>2.4</td>
<td>Gradients used in RP-HPLC analysis</td>
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<td>2.5</td>
<td>Biotin/streptavidin functionalisation</td>
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<tr>
<td>2.6</td>
<td>Maleimide reaction</td>
<td>34</td>
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<td>2.7</td>
<td>Automated western blot work-flow</td>
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</tr>
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<td>(3 - (4,5 - Dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) reaction</td>
<td>42</td>
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<td>2.9</td>
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