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Evaluation of Biological Biomaterial Properties using Microfluidic Systems

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Abstract

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Despite increased orthopedic biomaterial research activity over previous decades, relatively few novel biomaterials have made it to clinical use. This may partially be due to the inability of existing *in vitro* testing routines to sufficiently replicate the physiological environment, leading to potentially inaccurate assessments of a biomaterial's therapeutic potential. To address this, mathematical modelling and microfluidic design principles were assessed as possible supportive strategies to better improve the informativity of *in vitro* testing approaches.

Using principles of the Langmuir isotherm, a predictive computational model was constructed to capture the dynamics of protein and cell adhesion on a biomaterial surface, specifically on calcium-deficient hydroxyapatite, which is a synthetic biomaterial that is compositionally similar to the inorganic phase of the bone. The results demonstrated the success of the model at capturing the trends of the data, thereby indicating potential use as a predicative tool to assist with *in vitro* data interpretation.

Furthermore, attempts were made to improve the *in vitro* environment towards better physiological relevancy via the introduction of microfluidics, which is method of precise fluid control in micron-sized channels. For instance, the use of microfluidics allows for cell culture under more tissue relevant length scales, as well as the provision of a continuous media flow, which facilitates nutrient delivery and activation of mechanosensitive pathways through shear stress. Through development of such "Biomaterial-on-chip" microfluidic platforms, a general increase in cell viability and proliferation was seen when cells were cultured under flow. The effect of flow on other parameters such as material-induced ionic exchange, immunogenicity and mechanotransduction was also tested using the platform. By the culmination of the thesis work, the Biomaterial-on-chip platform was designed with inherent standardization, allowing for the *in vitro* testing of different biomaterials of varying shapes and properties under the same conditions in the same platform. All in all, the main conclusion from this thesis work is that cell response can largely differ depending on the chosen culture conditions, which therefore necessities careful consideration of environmental parameters prior to the start of an *in vitro* biomaterial evaluation study.

Keywords: Bone, Flow, Hydroxyapatite, In vitro, Modelling, On-chip, PDMS

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Atif, A.R.**, Lācis, U., Engqvist, H., Tenje, M., Bagheri, S., Mestres, G. (2022) Experimental Characterization and Mathematical Modeling of the Adsorption of Proteins and Cells on Biomimetic Hydroxyapatite, *ACS Omega*, 7: 908–920.

II. Carter, S-S.D., **Atif, A.R.**, Kadekar, S., Lanekoff, I., Engqvist, H., Varghese, O.P., Tenje, M., Mestres, G. (2020) PDMS leaching and its implications for on-chip studies focusing on bone regeneration applications, *Organs-on-a-Chip*, 2: 100004.

III. **Atif, A.R.**, Pujari-Palmer, M., Tenje, M., Mestres, G. (2021) A microfluidics-based method for culturing osteoblasts on biomimetic hydroxyapatite, *Acta Biomaterialia*, 127: 327-337.

IV. Carter, S-S.D., **Atif, A.R.**, Diez-Escudero, A., Grape, M., Ginebra, M.P., Tenje, M., Mestres, G. (2022) A microfluidic-based approach to investigate the inflammatory response of macrophages to pristine and drug-loaded nanostructured hydroxyapatite, *Materials Today Bio*, 16: 100351.

V. **Atif, A.R.**, Aramesh, M., Carter, S-S.D., Wang, H., Tenje, M., Mestres, G. (2023) A universal microfluidic platform for *in vitro* biomaterial evaluation, *Manuscript under review (Small)*.

All included publications are open-access and permitted inclusion into thesis.

Author's contributions

The author contributed to the enclosed articles in the following manner:

I. Part of Planning. Performed cell culture experiments. Wrote majority of the publication.

II. Part of Planning. Manufactured chips and performed chemical quantification of silicon leachate. Contributed to writing of publication.

III. Major contribution towards planning. Developed chip and performed all laboratory work. Wrote the publication.

IV. Part of Planning. Developed microfluidic device, prepared cement samples and assisted with drug quantification and protein imaging. Supervised MSc student working on the project. Contributed to writing of publication.

V. Major part of planning. Developed microfluidic device and performed all experimental work. Wrote the publication.

Other contributions

International and national conference and scientific meeting presentations are listed below. The presenting author is underlined.

I. **Atif, A.R.**, Carter, S.S.D., Pujari-Palmer, M., Tenje, M., Mestres, G. (2018) Bone cement embedded in a microfluidic device, *Micronano System Workshop (MSW)*, (Aalto, Finland), **poster presentation. Best poster winner.**

II. Carter, S.S.D., **Atif, A.R.**, Lanekoff, I., Tenje, M., Mestres, G. (2018) Tailoring the biocompatibility of the elastomer PDMS for on-chip applications. *Scandinavian Society for Biomaterials (ScSB)* (Gothenburg, Sweden), **poster presentation.**

III. Carter, S.S.D., **Atif, A.R.**, Lanekoff, I., Tenje, M., Mestres, G. (2018) Improving the biocompatibility of PDMS by improving its curing time and temperature. *European Organ-on-Chips Society Conference (EUROoCS)* (Stuttgart, Germany), **poster presentation.**

IV. **Atif, A.R.**, Pujari-Palmer, M., Tenje, M., Mestres, G. (2019) Evaluation of Ionic Interactions of Bone Cement-on-Chip, *European Organ-on-Chips Society Conference (EUROoCS)* (Graz, Austria), **poster presentation.**

V. **Atif, A.R.**, Pujari-Palmer, M., Tenje, M., Mestres, G. (2019) Quantitative evaluation of osteoblast proliferation and differentiation on a biomaterial in a microfluidic device, *European Organ-on-Chips Society Conference (EUROoCS)* (online), **oral presentation.**

VI. **Atif, A.R.**, Lācis, U., Tenje, M., Bagheri, S., Mestres, G. (2021) Modelling adsorption of proteins and cells on biomimetic hydroxyapatite, *European Society for Biomaterials (ESB)* (online), **poster presentation.**

VII. Carter, S.S.D., **Atif, A.R.**, Pujari-Palmer, M., Barbe, L., Tenje, M., Mestres, G. (2021) Biomaterials-on-chip: an alternative method to screen the biological properties of biomaterials. *European Society for Biomaterials (ESB)* (online), **oral presentation. Best oral presentation award.**

VIII. **Atif, A.R.**, Pujari-Palmer, M., Tenje, M., Mestres, G. (2021) Influence of flow in the adhesion and proliferation of cells on hydroxyapatite integrated in a microscale culture, *Scandinavian Society for Biomaterials (ScSB)* (online), **oral presentation.**

IX. **Atif, A.R.**, Aramesh, M., Carter, S.S.D., Tenje, M., Mestres, G. (2022) A microfluidic device for universal assessment of biomaterials in vitro, *Swedish Microfluidics Network (SMILS)* (Uppsala, Sweden), **poster presentation.**

X. **Atif, A.R.**, Carter, S.S.D., Wang, H., Engkvist, H., Tenje, M., Mestres, G. (2022) A universal microfluidic platform for in vitro biomaterial evaluation, *Scandinavian Society for Biomaterials (ScSB)*, (Jurmula, Latvia), **oral presentation. Travel grant awarded.**

Other:

VI. Carter, S.S.D., **Atif, A.R.**, Lanekoff, I., Tenje, M., Mestres, G. (2017) Biomaterial-on-chip, *Uppsala Biomaterials and Bioengineering Meeting* (Uppsala, Sweden), **poster presentation. Best poster award.**

VII. **Atif, A.R.**, Pujari-Palmer, M., Tenje, M., Mestres, G. (2019) Optimization of flow rate for improved cell proliferation in cement-on-chip platform. *Uppsala Biomaterials and Bioengineering Meeting* (Uppsala Sweden), **oral presentation.**

VIII. Carter, S.S.D., **Atif, A.R.**, Pujari-Palmer, M., Barbe, L., Tenje, M., Mestres, G. (2021) Developing biomaterial-on-chip systems to screen the biological properties of biomaterials. *Uppsala Biomaterials and Bioengineering Meeting* (Uppsala Sweden), **oral presentation.**

Contents

Abbreviations

[Si]	Silicon concentration
ABS	Acrylonitrile butadiene styrene
ALP	Alkaline phosphatase
AM	Acetoxymethyl
BMP-2	Bone morphogenetic protein 2
BSA	Bovine serum albumin
CDHA	Calcium-deficient hydroxyapatite
CMFDA	5-chloromethylfluorescein diacetate
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FBR	Foreign body reaction
FFF	Fused filament fabrication
HA	Hydroxyapatite
hMSC	Human mesenchymal stem cell
ISO	International Organization for Standardization
LDH	Lactate dehydrogenase
OCN	Osteocalcin
PDMS	Polydimethylsiloxane
PLA	Polylactic acid
qRT-PCR	Quantitative real time – polymer chain reaction
SLA	Stereolithography
Ti	Titanium
TNF-α	Tumour Necrosis Factor alpha
UBoC	Universal biomaterials on chip
UV	Ultraviolet

Introduction

Scope

Within the coming decades, a significant proportion of the global population will be above the age of 65,[1] an age span where intrinsic tissue reparability is markedly reduced. As such, an increase in implant adoption is expected due to greater fracture incidence among the overall population.[2] To meet expected demand, multiple state-of-the-art avenues of research into implant biomaterial science are actively being explored. A number of different types of biomaterials and their orthopaedic usage are discussed in **Chapter 1 "Biomaterials: Introduction and Background"**. Nevertheless, the translation of novel biomaterials into the clinical landscape has proven to be challenging due to multiple factors. Such difficulties include attainment of regulatory endorsement,[3] high developmental cost,[4] insufficient scaffold bioactivity,[3] and lack of preclinical *in vivo* model informativity.[5] Likewise, *in vitro* validation has also been criticized, as studies have reported low correlation between *in vitro* assessments,[6–9] as well as against corresponding *in vivo* results.[8,10,11] Alternatives such as *in silico* analytics, where mathematical descriptors of physiological processes are formulated, have arisen as techniques to increase pre-clinical predictive power.[12–14] Issues pertaining to clinical translation and possible countermeasures are described in **Chapter 2 "Biomaterial Evaluation"**.

One potential improvement towards effective biomaterial clinical translation is the development of more representative *in vitro* evaluation platforms that better capture the modalities of the *in vivo* environment. This may be achieved through microfluidics, which enable control of small volumes of fluids.[15] An introduction into microfluidics and its benefits is provided in **Chapter 3: "Microfluidics"**. Via microfluidics, cells can be cultured in one or more compartments, within dimensions more reminiscent to *in vivo*. Continuous nutrient supply and waste removal are also provided to the culture, with mechanical cues generated via a shear stimulation by the applied flow. The technique has been instrumental in developing organ-on-chip systems through features such as multiplexing, precise fluid delivery and sampling.[16]

Aim and Research Questions

The main aim of this thesis is the methodological investigation of alternative *in vitro* evaluation techniques for orthopaedic applications, with the main focus being placed on microfluidics. The feasibility of mathematical-based analysis was briefly explored as a supporting technique. The research questions that were studied in the presented papers are:

- Can mathematical modelling supplement *in vitro* biomaterial characterization?
- Does leaching of polydimethylsiloxane (PDMS) by-products influence cell culture proliferation and differentiation when used in microfluidic chip production?
- Does constant perfusion affect *in vitro* cell proliferation and differentiation on biomaterials?
- Can an on-chip microfluidic system be suitable for the study of the biological behaviours of cements of differing properties?
- Can microfluidics be used for biomaterial characterization of behaviours such as protein adhesion and drug release?
- Can a single platform be used for standardized flow-based *in vitro* characterization of different biomaterials of varying properties?

In **Paper I**, a mathematical model based on the Langmuir isotherm was developed for the evaluation of protein and cell adhesion dynamics on Hydroxyapatite (HA), which was used as a model biomaterial. In **Paper II**, initial steps into microfluidics production were made, where the focus was on the characterization and validation of PDMS as a suitable substrate for chip fabrication. Using the information gleaned in papers I and II, a microfluidic device incorporating hydroxyapatite (HA-on-chip) was constructed to evaluate the effect of flow on cell culture on HA as opposed to static culture and this is outlined in **Paper III**. In **Paper IV**, the applicability of the HA-on-Chip was further demonstrated through an analysis of micro-structured HA, specifically of protein penetration and immunogenicity under dynamic conditions of flow. The drug release ability of both formulations under flow was also assessed as a feature of the publication. Finally, in **Paper V**, a universal microfluidics system was developed that could assay a wide variety of biomaterials under an applied flow for properties such as biocompatibility, mechanosensitivity and cell-cell interactions. An overview of each publication can be found in **Chapter 4. Summary of Included Publications**.

Chapter 1. Biomaterials: Introduction and Background

1.1. Bone Tissue

To better understand biomaterial functionality and its associated challenges, one has to first understand the physiological context of tissue that the biomaterial will be implanted into. In this section, focus is placed on orthopaedic implants, with bone being the target tissue for implantation. Bone is a stiff tissue that makes up the skeletal system and provides a supportive and protective framework for the body. Some critical bone functions include physical maintenance of bodily integrity and mineral ion storage.[17] Generally, bone is a composite material made up mainly of an inorganic mineral phase, an organic proteinaceous phase and water, which in this arrangement gives bone unique properties of mechanical strength, viscoelasticity and fracture resistance.[18–20] Bone mineral can be epitomized as stoichiometric hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), but it is actually a more complex structure with substituted trace elements into the lattice such as CO_3^{2-}, Na^+ and Mg^{2+} leading to non-stoichiometric configurations and lower Ca/P ratios.[20,21] As for the organic phase, it is predominantly composed of type 1 collagen,[22,23] and other supportive proteins such as fibronectin, osteonectin and osteocalcein.[23,24] Within the body, bone can either be found in a compact or cancellous configuration. Compact bone makes up 80% of the total bone content in humans and is arranged in repeating units of parallel structures known as osteons, which consist of mineralized concentric lamellar rings with hollow vascularized and innervated central tubes known as Haversian canals (Figure 1A).[25] Osteons are also perpendicularly connected to each other and eventually to the periosteum via Volkmann canals (Figure 1A).[25] Compact bone is comparably stiff and generally acts to support loading on the bone framework. On the other hand, cancellous bone, which makes up the remaining 20% of bone mass, is less dense and consists of highly interconnected vascularized cavities connected via canaliculi. Due to its higher porosity, cancellous bone is more elastic and receptive towards adaptive mechanical loading.[19,25]

While perhaps counterintuitive, bone is a highly dynamic tissue undergoing several anabolic and catabolic cycles throughout life. Specifically, the human skeleton is effectively fully replaced every 10 years.[26] The underlying process

is known as bone remodelling and is the driving force for intrinsic bone healing and ability to autonomously repair microfractures.[17,19] The process is tightly spatiotemporally controlled and is activated as a result of stimuli such as mechanical loading,[27] microcrack formation,[19,27] and osteocyte injury.[19] After appropriate stimuli, osteoclast activity is enhanced, which then acts to dissolve the bone matrix back into free calcium and phosphate ions in a localized area through acidification and enzymatic degradation.[25,28–30] Once the damaged area has been fully eroded, osteoclast activity is gradually dampened and replaced with increased osteoblast activity.[19] Osteoblasts secrete and fill the cavity with osteoid, which is a nonmineralized mix of collagen and other proteins, such as osteocalcin and osteopontin.[19,31] After the deposition of osteoid, recruited osteoblasts undergo apoptosis,[32] become trapped in matrix and mature into osteocytes,[31,32] or cover the osteoid surface and remain as bone lining cells.[32] The cells lining the osteoid then aid in mineralization via alkalization of the osteoid local area through removal of protons,[33] as well as transmembrane transport of calcium and phosphate ions.[34,35] The remaining osteocytes confine into small lacunae and form dense interconnections through cell processes extending through exceptionally narrow canaliculi (~ 1 µm diameter) which altogether make up the lacunar-canalicular system (Figure 1A).[31,36] When bone is compressed or strained, tissue fluid is squeezed through the canaliculi, imparting a high degree of strain on osteocytes (Figure 1B). These fluid movements trigger intracellular signalling mechanisms which culminate in modified osteoblast or osteoclast activity, thereby shifting the balance between bone anabolism and catabolism.

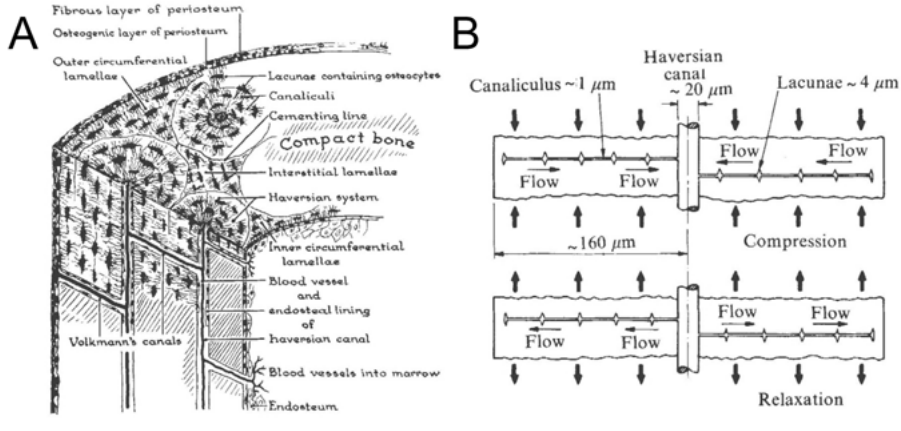

Figure 1. A) Schematic of compact bone transversal-section. B) Depiction of fluid movements in canalicular-lacunar system as a result of compression and relaxation. Adapted from Munro, 1977.[37]

For larger, more complex fractures, bone remodelling alone is insufficient and medical intervention through surgical auto- or allografting is generally

required to aid healing. Autografts are considered the gold standard and entail the extraction of patient-derived tissue and subsequent re-implantation in the trauma site within same patient. Commonly, the iliac crest is used as a source for graft collection.[38] Autografts display maximal biocompatibility with the patient and are considered osteoinductive and elicit new bone formation.[20] Drawbacks to the use of autografts include the need for extra surgical procedures which may put excessive strain on the patient, limited availability and possible insufficiency when used in exceptionally large trauma sites. Allografts partially solve the issue, but are also limited in supply and are relatively expensive.[20]

1.2. Biomaterial Development

A biomaterial is a substance that is able to ameliorate or potentially recover diseased tissue function.[39] Within the context of bone and hard tissues, some applications of biomaterials include usage as supportive matrices in dental fillings, bone screws and bone plates.[39] In this thesis, pre-existing established biomaterials were used as models, but we believe it to be useful to briefly clarify the biomaterial development process. In essence, the development of orthopaedic biomaterials over the last century can generally be separated into distinct phases, each driven by different rationale (Figure 2). Initially, the main motivation was the development of substrates with mechanical properties akin to bone while maintaining inertness, resulting in a lack of harmful tissue interactions.[40] However, as a consequence of inertness, subpar interaction between the material and the existing cellular architecture may result, leading to possible fibrous capsule formation around the implants and weak tissue adhesion, culminating in poor long-term prognosis. To address this, biomaterial development over the years has been expanded to include bioactivity as a desirable feature, with evocation of therapeutic responses to bodily afflictions as a main aim.[41] Bioactivity entails a chemical interaction between the biomaterial and the physiological tissue environment.[42,43] Such interactions are generally uncontrolled and dependent on passive processes such as diffusion.[43] An example would be dissolution, where the material is degraded within the body over time, as is the case with calcium phosphate cements and magnesium-based implants.[44,45] Moving on, the next generation of biomaterials were developed to induce responses only to particular stimuli, thus increasing the efficacy of interaction and extending response duration. Examples include polymers that undergo Young's moduli alterations as a response to mechanochemical stimuli.[46,47] All in all, the ultimate goal of biomaterial development is to foster the creation of a suite of materials compatible for use in various patients which have a minimal need for revision surgeries multiple years after implantation.

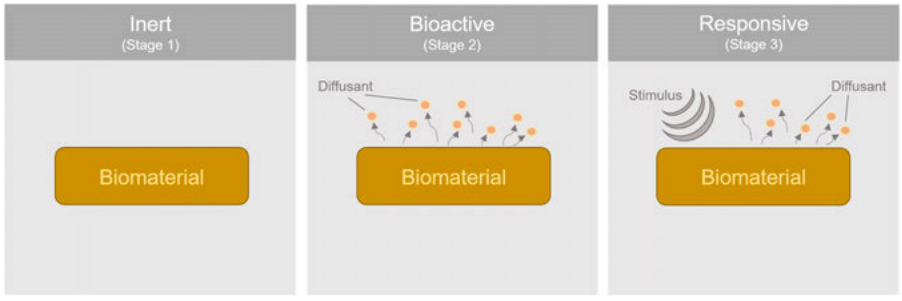

Figure 2. Overview of biomaterial development process, with specific focus on inert, bioactive and responsive phases.

1.3. Immunogenicity and Inflammatory Processes

Following biomaterial implantation, a series of critical steps occur that define the host response to the implant. The process is known as the foreign body reaction (FBR) and is of relevance to **Paper IV**, where immunogenicity of bone cement is investigated.[48] The FBR process is described briefly herein. Initially, there is a rapid nonspecific recruitment of plasma proteins to the implant surface (*e.g.* albumin, fibronectin). The adhered protein population will largely depend on the surface properties of the implant, with factors such as surface charge and contact angle being of pertinence. Due to blood vessel damage caused by surgical implantation, platelets will initiate a clotting cascade and form a supportive matrix around the implant and connective tissue through means such as binding to collagen fibrils. Other immune cells will meanwhile be recruited towards the site, triggering inflammation. Monocyte-derived macrophages are of particular interest as they display plastic behaviour depending on the stimuli encountered at the surgical site.[49,50] Macrophages then release proteases and secrete cytokines that may influence bone remodelling,[51,52] as well as phagocytose contaminant microbes and may eventually mature into multi-nucleated foreign body giant cells (FBGCs).[50] Subsequently, fibroblasts will converge onto the inflammation site and differentiate into proto-myofibroblasts, which maintain tissue integrity through the formation of cell-matrix adhesion links.[50,53] Afterwards, cells will further differentiate into myofibroblasts and then vigorously lay down new extracellular matrix (ECM) that will serve as a framework for forthcoming regeneration processes.[50,53] Several strategies exist to improve implant integration and compatibility with FBR. Application of biocompatible coatings on bulk materials can, for example, enhance hydrophilicity which promotes non-specific protein adhesion and interaction with immune cells.[54]

1.4. Biomaterial Types

1.4.1. Hydroxyapatite

Calcium phosphate cements (CPCs) are a class of biomaterials that display optimal properties with regards to implantation that improve upon their progenitors: acrylic-based bone cements.[55] Prior to implantation, CPC precursors are mixed with water, forming a paste that can be injected into the implant site, where the material is able to set and harden through a dissolution-precipitation reaction.[55,56] Unlike acrylic bone cements, the CPC setting reaction is not exothermic, thus maintaining tissue at physiological temperatures and limiting damage to tissues from excessive heat build-up.[55] Depending on the specific composition and inherent solubilities, CPCs are able to dissolve and degrade in the body, eventually being replaced by *de novo* bone tissue.[44] The material can degrade by physical means, but can also be processed through cell-dependent mechanisms in a process known as resorbability.[44] In addition, interjoined porosity can be introduced into CPCs through techniques such as gel casting or foaming, which increase the overall surface area of interaction with the tissue and plasma proteins.[20,57–59] Nevertheless, at particularly high porosities, a liability of CPCs as implant materials is a low degree of mechanical strength which limits its usage to non-load bearing areas of the skeletal system.[55] Based on these properties, CPCs have found usage in orthopaedic and dental applications, such as tooth fillings and bone defect site packing.[20,55]

Hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) is a type of apatite calcium phosphate cement that resembles the inorganic bone phase in terms of chemistry, crystal size and shape.[55] Depending on the specific composition, HA displays several relevant properties such as biocompatibility and bioactivity, which mirror the properties of physiological bone tissue.[20,55] Specifically, bone mineral is non-stoichiometric and ion-substituted and is best approximated with a particular type of HA called calcium-deficient hydroxyapatite (CDHA; $Ca_{10-x}(HPO_4)(PO_4)_{6-x}(OH)_{2-x}$, *where* $0 \geq x \geq 1$) that can be synthesized via a low temperature hydrolysis reaction from an α-tricalcium phosphate (α-TCP) precursor in an aqueous ionic soultion.[20,57,60,61] The non-stoichiometric nature of CDHA also leads to ion reactivity, where Ca^{2+} and PO_4^{3-} ions are absorbed from and released into a solution in contact with the material, respectively.[62,63] Such changes in ionic composition are considered bioactive and may have effects on cell culture, which is further explored specifically in **Paper III**. In terms of cellular properties, CDHA is osteoconductive and able to promote mesenchymal cell expression of critical bone anabolic genes such as *BMP-2*, *OCN* and *ALP* necessary for osteogenic fate differentiation and encouragement of bone healing.[58,64] Due to lower Ca/P ratios,[61] CDHA is more soluble than HA and is more compliant towards osteoclast-induced resorption and

degradation,[65] subsequently being replaced with bone tissue laid down by osteoblasts. As such, CDHA is able to serve as a scaffold for bone regeneration.[20]

1.4.2. Medical-Grade Titanium

Metallic biomaterials first found usage as clinical implants in the 1920s, with stainless steel being the initial material of choice due to its high mechanical strength and overall inertness.[1] However, the main challenge of using such implants was an issue of long-term corrosion due to mechanical friction and tissue fluid contact. Not only would friction wear down and damage the implant, resultant leached metal ions may exceed tolerable bodily concentrations and subsequently exert detrimental effects on biological tissue.[39,66] Over the coming decades, other metals such as molybdenum (Mo), nickel (Ni) and vanadium (V) have been alloyed with steel to attempt to further improve corrosion resistance.[39] Eventually, titanium (Ti) and its alloys (such as Ti_6Al_4V) were adopted as implants for load-bearing bone tissues, such as hip and leg joint replacements.[1] In addition to enhanced corrosion resilience, Ti is less dense than steel and results in lighter implants with higher specific strength.[39] Ti is also considered more elastic than steel and better approaches the elastic modulus of bone (13 GPa), reducing stress shielding by allowing *in vivo* bone to experience a greater proportion of applied forces (such as during walking), leading to increased remodelling and reduced bone atrophy.[67,68] Moving on, a critical property of Ti is the ability to form strong direct connections to the bone tissue in a process known as osseointegration,[69] helping to secure the implant in place and increase operational lifetime. Attempts to enhance osseointegration were investigated, such as the use of patterned surface texturization, which was observed to promote osteoblast cell attachment and proliferation *in vitro*.[70] Due to the aforementioned properties, Ti is commonly used in load-bearing orthopaedic applications such as hip-joint implants and dental implants.[69,71]

1.4.3. Hydrogels

Hydrogels are materials made up of insoluble hydrophilic polymer chains that are able to take up large volumes of water when placed in aqueous solutions.[72,73] Depending on the functional groups present on the polymer chains, different bonding modalities are possible, resulting in either physically-bound gels held by weak intermolecular forces (reversible) or covalently-bound (permanent) gels.[73] Hydrogels have been used as the basis of 3D-cell culture and tissue engineering to replicate ECM, with cell culture performed in the gel more akin to the *in vivo* situation due to a 3-dimenional growth scaffold that promotes nutrient and gas exchange.[73–75] High intrinsic gel porosities created via polymer chain repulsion maximize water penetration and swelling of the gel bulk,[73] concurrently leading to low stiffnesses which alleviate

inflammation and mechanical damage to surrounding tissues after implantation.[76] As such, hydrogels are considered an ideal medium for the delivery of therapeutic agents such as encapsulated cells or drug molecules. Cells interact and interface with the gel through linker plasma-membrane proteins called integrins, which act as anchors for processes such as mechanosensation.[75] Integrins are receptive to a wide range of ligands, with a prominent example being the RGD peptide sequence, which is found in matrix proteins such as fibronectin and fibrinogen and is commonly used as an attachment peptide for patterning hydrogel substrates.[77,78] Nonetheless, there is considerable choice in polymer gels, with collagen, fibrin and polyethylene glycol (PEG) gels being common examples. In **Paper V**, fibrin was used as a representative polymer to demonstrate the capabilities of a microfluidic device for hydrogel assessment. Fibrin is normally formed *in vivo* during the wound-healing process when damage to vasculature triggers the conversion of prothrombin into its active form; thrombin.[79] Thrombin then catalyses the conversion of soluble fibrinogen in the blood into insoluble fibrin, which plugs the wound-site, prevents further bleeding and acts as a framework for further healing processes.[79] It has therefore found usage in *in vitro* systems for study of angiogenesis and microvasculature formation.[80] Some advantages of fibrin include direct procurement from the patient and direct use as autologous implants.[81] Similarly to CDHA, fibrin precursors can also be injected directly as liquid blends into the trauma site, effectively filling the shape of the defect before setting. However, the low stiffness of fibrin gels (and hydrogels in general) limits usage to small non-load bearing defects. To counteract low toughness, production of fibrin composites has been attempted to better approach mechanical properties of physiological bone.[82]

Chapter 2. Biomaterial Evaluation

2.1. *In vitro* vs *In vivo* Analysis

Before novel biomaterials are certified for clinical use, satisfactory performance in animal models is required, which today is still considered the gold standard for biomaterial assessment. However, it is impractical in terms of cost, time and ethics to evaluate a disproportionate selection of promising materials *in vivo*. Instead, the most relevant *in vivo* environmental processes were adapted into an *in vitro* assay format to enable more effective biomaterial screening and isolation of candidates that display favourable therapeutic responses. Due to the complex and multi-layer interactions that exist in the physiological environment, it is difficult to create an *in vitro* technique that is fully representative. As such, approximations are taken, usually involving the use of cell line cultures on biomaterial samples in static well plates.[83] Prior to acceptance, biomaterial candidates require validation through a checklist of multiple *in vitro* assessments, with one individual test being insufficient to determine biocompatibility.[83] According to ISO 10933-1, several parameters are investigated such as acute and chronic toxicity, irritation and hemocompatibility.[84] There are three main types of standardized morphological tests that are used to determine biocompatibility: direct contact, agar diffusion and elution.[48,83] In order to maintain impartial comparison between biomaterials, the tests are ideally performed using the same biomaterial surface area, size, cell line and seeding density and other parameters to ensure replicability and comparability.

While the use of *in vitro* studies strikes a balance in terms of convenience and feasibility, it may lead to imperfect conclusions. For instance, within the context of bone implants, there was an overall lack of covariance (58%) between *in vitro* and *in vivo* cell study outcomes.[8] Bone formation *in vivo* is morphologically dissimilar to *in vitro* (particularly in 2D) bone, which is more analogous to bone-like nodules that fail to capture the organized geometry of the osteon.[31,85] In particular, *in vitro* osteoblast-precursor cultures displayed uncharacteristic physiology such as sclerostin overexpression,[31,86] excessive cell-cell connections,[31] as well as discrepancies in hypoxia response.[31,87] Another discrepancy can be seen, for example with intravitreal pharmacokinetic drug injection outcomes between *in vitro* and *in vivo* models.[10] In addition, in the case of primary cells (such as mesenchymal cells used in **Paper II**), there

is significant phenotypic differences between naïve and isolated cells due to factors such as donor variation and culture environment,[6,7,9] thus reducing predictive power. As such, care needs to be taken when drawing conclusions from purely *in vitro* data and there is a demonstrated need for improved culture systems that are better predictive of *in vivo* outcomes.

2.2. Cell Culture

Cell culture involves the cultivation of cell populations outside of the body, which can be used to assess cell response to different factors or materials. The exact culture formats can vary, but can generally be subdivided into direct and indirect arrangements. Specifically, direct culture implies the seeding of the cells directly on the material, whereas indirect methods constitute cell culture off the material surface (for instance, by using Transwell inserts or in medium that was preincubated with the biomaterial beforehand). These methods are illustrated in Figure 3. Different parameters are highlighted in the two formats, with direct culture for example emphasizing the effect of microstructure on cell attachment, while indirect set-ups underscore soluble factor cell-material interactions. In this thesis, murine MC3T3-E1 pre-osteoblasts, L929 fibroblasts and RAW 264.7 macrophages have been utilized in **Papers I through V** as they are commonly used for biocompatibility *in vitro* evaluations.[84,88,89] Generally, a particular cell line is chosen depending on the *in vivo* parameters that would be most relevant for the implant properties to be tested.

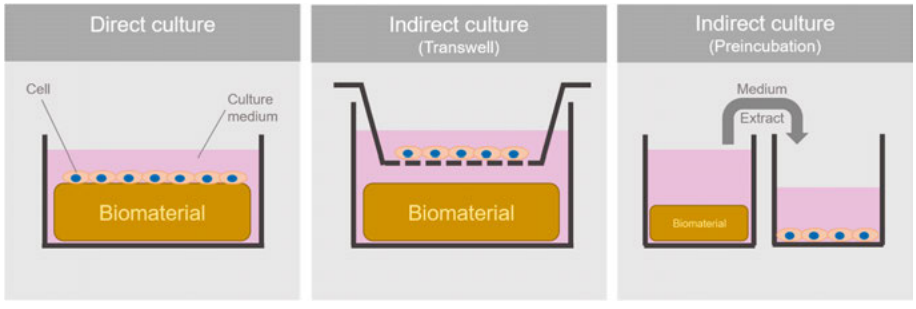

Figure 3. Illustration of different culture techniques possible in the context of biomaterial *in vitro* evaluation.

During *in vitro* culture, biomaterial-induced cell responses can be investigated through a variety of techniques. A common method involves the staining of different cell locales to visualize organelles or confirm cellular status. An example would be the use of calcein to confirm cell viability by staining of cytoplasm. Dyes that are retained longer in cells can also be used and allow the culture to be continuously followed, either to investigate proliferation dynamics and/or to monitor cell motility and migration, with an example of such a

dye being Green CMFDA. These dyes work via first being added in solution to the cell culture in a non-fluorescent format conjugated with an acetoxymethyl (AM) group. While bound to the dye, AM sterically hinders chelation of divalent ions, which would normally activate the fluorescent properties of the dye. In addition, AM allows the dye to diffuse freely through the cell membrane to reach the cytoplasm, where cytoplasmic esterases are able to cleave the AM group. Subsequently, fluorescence activates via chelation and the dye becomes cell-impermeant, causing it to be trapped in the cell and leading to amplification in signal. Cell viability dyes are usually counter-stained against nuclear dyes that show the morphology and localization of the nucleus (*e.g.* Hoechst) or presence of dead cells via intercalation of cell-impermeant dyes with DNA in the nucleus (*e.g.* propidium iodide). Within this thesis, cell viability staining was frequently used within the majority of the included papers (**I, III, IV and V**). Furthermore, staining is not only limited to cells, but can also be extended to proteins as well. Proteins can be chemically tagged with a fluorescent label, allowing the localization of the protein to be determined. This strategy was exploited in **Paper IV** to estimate protein permeation into the bulk of an HA sample.

An important property quantified on multiple occasions in this thesis is the degree of proliferation cells experience when cultured under specific conditions. In **Paper III and V**, proliferation was quantified using cell staining procedures as described earlier, followed by enumeration of viable cells over a range of time points. In addition, an alternative method also used in **Paper III and V** of this thesis for proliferation analysis was the colorimetric quantification of Lactate Dehydrogenase (LDH) activity, which is a cytoplasmic enzyme found in most body cells.[90] Through cell lysis, the LDH enzyme is released and can subsequently be used to catalyse the reduction of a tetrazolium salt, resulting in an opaque soluble product,[91] whose degree of light absorption (at a specific wavelength) can be correlated to total cell content. While effective, this technique is performed under the assumption of constant LDH expression of all cells for given culture conditions. As such, it is ideal to validate LDH quantification using other assays (such as imaging) to ensure accurate results.

A relevant protein to the included research work in this thesis is alkaline phosphatase (ALP), which is indicative of the osteoconductivity (and perhaps osteoinductivity) of the implant when cultured in the orthopaedic context *in vitro*. Serum levels of ALP are also prognostic of new bone formation *in vivo*.[92,93] Briefly, ALP is a plasma membrane-bound protein commonly found in multiple body tissues and within the bone is highly expressed by osteoblasts.[94] ALP has important roles in mineralization and acts to increase local concentrations of free inorganic phosphate.[94] ALP was assessed in **Paper II** and **III** as a marker for osteoblast differentiation under the effects of leached PDMS molecules and perfused cell culture, respectively. Alternatively,

collagen, osteocalcin and osteopontin are substitute osteogenic markers that are relevant to the bone remodelling process and can be analysed for osteoconductivity.[31] Ideally, more than one marker should be analysed to ensure a more accurate picture of the differentiation process.

Quantitative real-time polymerase chain reaction (qRT-PCR) is a technique where the expression of target genes can be amplified and quantitatively investigated. Specifically, the technique can be used to compare differential expression of genes of interest as a function of different cellular treatments. For instance, in **Paper II**, qRT-PCR was used to investigate key osteogenic gene expression profiles of murine mesenchymal stem cells after exposure to elevated levels of Si^{+} ions. Rather than just examining products of gene transcription, investigation of downstream translation products through direct measurement of secreted protein levels may further expand understanding of expression dynamics. Enzyme-linked immunosorbent assays (ELISA) use antibody-based interactions to bind the target protein and indicate abundance via colorimetric enzymatic assays. For example, in **Paper IV**, ELISA was used to quantify Tumour Necrosis Factor alpha (TNF-α) concentrations secreted by RAW 264.7 after exposure to samples of CDHA of varying microstructure. For optimal assessment, care needs to be taken in regards to protein release timings and rates, as well as protein half-life in culture medium after release.

2.3. *In silico* Analysis

In silico analysis refers to the use of computational models and mathematical equations to describe biological behaviour as a function of representative factors. The technique may assist in the creation of new hypotheses,[12] and can assist with chemometric drug discovery analytics.[13,95] As the *in vivo* implant environment is complex and composed of a multitude of interacting entities and factors, it is difficult to create a model that is able to capture all of the underlying physiological factors.[12] Instead, it is more worthwhile to focus on a subset of interactions that are most vital to the prognosis of the implant. A rudimentary example of *in silico* analysis was used in **Paper I**, where protein and cell biomaterial-interaction dynamics were modelled based on the Langmuir isotherm. A comprehensive dataset of biomaterial cell responses, gathered from either *in vitro* or *in vivo* sources, is generally required to perform in-depth *in silico* analysis. For example, to better understand the relationship between material topography and osteoblast differentiation, Hulshof *et al.* built a computational model to correlate high-throughput *in vitro* experiments performed on a large variety of micro-structured surfaces.[13] The model developed by Hulshof *et al.* identified a selection of surfaces that displayed optimal osteogenic behaviour, which in further *in vivo* testing, showed performance comparable to established medical implant surfaces.[13] Another example,

developed by Wolf *et al.*, was to build an *in silico* model of macrophage response, where ELISA was performed using the supernatant of different matrix samples for a selection of representative proteins such as Interlukin-6 (IL-6) and Matrix metallopeptidase-9 (MMP-9), both of which are proteins involved with inflammation and wound repair.[14] Using the Wolf *et al.* model, a unique protein release profile was generated for each tested biomaterial.[14]

Chapter 3. Microfluidics

3.1. What is Microfluidics?

Microfluidics is the study and control of fluid dynamics within sub-micron channels and features,[15] with mass transport and fluid mixing being examples of relevant behaviour. At the macroscale (such as meter scale), fluid flow is heavily dependent on the action of volumetric forces with inertia playing a major role. However, as length scales are reduced into the micron range and below, surface forces begin playing a more substantial role in determining fluid flow,[96,97] with phenomena such as capillary and electrophoretic forces establishing greater relevance. Reynolds number (Re) can be used to predict fluid behaviour by measuring the ratio between inertial and surface forces and is given by the formula below:[96]

$$Re = \frac{\rho \cdot u \cdot L}{\mu}$$

Where ρ is density, u is fluid velocity, L is characteristic length and μ is dynamic viscosity. Lower Reynolds numbers indicate a dominance of surface forces and a resultant laminarity of the flow profile (Figure 4).[96,98] As inertia-induced nonlinearity is reduced,[96] flow becomes more predictable, allowing more precise measure of flow-dependent parameters, such as ionic leaching and drug release, as was investigated in **Papers II**, **III, IV** and **V**. The laminar nature of the flow implies that fluid laminas are able to flow abreast as parallel layers without mixing,[99,100] and this is the basis of the coculture performed in **Paper V** in the UBoC device. In addition, as diffusion is the only means of lateral particle movement under laminar conditions, this arrangement is, for example, suitable for gradient generation of signalling cues or pharmaceutical agents.[101]

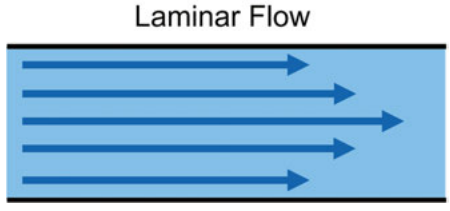

Figure 4. Depiction of laminar flow profile generated in typical microfluidic setups. Arrows indicate direction and magnitude of flow.

Within the biological context, microfluidics offers several benefits. The first is compartmentalization, where individual analytes or reactants can be stored in separate small compartments on chip, thereby enabling parallelization and miniaturization of common assays such as polymerase chain reaction (PCR), cytometry and immunoassays.[99,102,103] Smaller compartmental volumes also imply less reactant usage, with a concomitant reduction in reaction times and reagent costs.[98,104] Compartmentalization is also beneficial for direct cell culture, as different cell types can be precisely localized and cultured together on-chip in defined areas,[100,105] thereby emulating the tissue organization for better drug evaluation.[106] Cultured cells in microfluidic set-ups also receive a continuous supply of nutrients provided under flow,[106] ensuring constant optimal levels. Flow also provides mechanical stress conditioning to cultured cells,[107] which is of particular importance for mechanosensitive cells such as vascular endothelial cells or osteocytes.[108,109] Considering these advantages, it is not surprising to see microfluidics play a major role in the development of Organ-on-chip systems for better *in vitro* assessment.

3.2. Fabrication and Setup of Microfluidic Devices

3.2.1. Materials for Microfluidic Devices

Nowadays, there is great flexibility in the choice of material and tools for microfluidic device fabrication. Sub-micron feature generation for microfluidics was pioneered in the late 1960's using micromachining methods initially developed for integrated circuit fabrication,[110] where usage of silicon and glass wafers is common.[111] As glass is transparent and bioinert, it found particular relevance as a substrate for cell culture. Glass offers multiple other advantages, including a high degree of stiffness that makes it compatible with high aspect ratio fabrication and more amenable for automated, high-volume production.[112] In particular, the rigidity of glass served an important role in **Paper V,** where it was critical to ensure mechanical sealing of the device. Finally, glass is hydrophilic and allows for easy filling of glass devices using aqueous media, avoiding bubbles in the process.

In order to overcome the restrictive costs and tools needed to process glass, polymeric materials have been used instead, particularly for rapid-prototyping applications.[113] The most popular of these is PDMS, which is an optically clear, flexible silicone elastomer that is commonly used for the development of organ-on-chip systems due to multiple inherent advantageous features.[114] Most importantly, PDMS is biocompatible and transparent, allowing for live monitoring of cell cultures.[106,115] It is also gas-permeable,[115] permitting oxygenation and buffering of culture medium inside the chip when placed in an incubator. Finally, PDMS is cheap and allows for multiple rounds of iterative prototyping at a low cost, thereby enabling cost-effective design and validation phases.[116] However, there are some disadvantages with using PDMS, with a main one being its hydrophobicity, which makes initial loading of chips with aqueous media and circumvention of trapped bubbles difficult. In addition, the hydrophobic nature of PDMS may cause small molecules to be adsorbed onto the PDMS, thus potentially interfering with processes such as protein quantification or immunoassays.[115,117] Furthermore, while aiding gaseous exchange, the gas permeability of the PDMS substrate also allows for fluid evaporation, which may harm cell cultures if not accounted for. Finally, despite its biocompatibility, PDMS may display leaching behaviour that affects certain cell lines, as is described in **Paper II** included in this thesis.[117]

3.2.2. Rapid Prototyping of Microfluidic Chips

Soft lithography is an umbrella term for a collection of techniques, such as elastomer casting and hot embossing, that involve pattern transfer onto "softer" substrates through mechanical means.[118] These techniques were developed to bypass the prohibitive cost, expertise and cleanroom access needed for photolithography.[116] Of particular interest in this thesis is elastomer casting (such as PDMS) and this will be discussed below. Briefly, the technique involves the casting of a liquid prepolymer against a mould containing a positive-relief of the desired pattern. After casting, the polymer is cured at an elevated temperature, leaving a negative impression of the mould pattern on the resultant elastomer surface. Moulds can be re-used multiple times, allowing for cheap, simple and reproducible production, highlighting the potential of the technique for prototyping applications.[116] PDMS soft lithography was used in **Paper II** and **III** to manufacture microfluidic chips.

Moulds for soft lithography can be prepared through different means. While not used in this thesis, a common method that is particularly useful if highly-resolved moulds are needed (down to nm-range) is photolithography.[116] Succinctly, silicon or glass wafer substrates are generally used and the first step involves deposition of a sacrificial layer (photoresist) onto the wafer through techniques such as spin-coating.[119,120] Photoresists (such as SU-8) are a class of organic molecules with solubilities that are sensitive to short wavelength

light (*i.e.* UV).[121] A UV light source is shone through a mask corresponding to the desired pattern, striking only areas on the resist which are unprotected by the mask, thus selectively altering the solubility of exposed areas. Exposed photoresist is then washed away using the appropriate developer solvent and the wafer is then baked for a few minutes, setting the positive relief pattern on the wafer. In this state, the wafer is now ready for use as a master mould for soft lithography.

Another related strategy is xurography, which is a technique that enables the generation of patterned PDMS substrates in a rapid, mould-free manner, thereby skipping the laborious and expensive steps involved with cleanroom fabrication.[122] The method works via using a cutter plotter device to carve the desired pattern on a thin PDMS sheet using a Computer-Aided Design (CAD) file as reference.[122,123] While far from being able to generate the resolutions possible with photolithography, xurography can generate structures down to 20-200 μm,[122,124] which may be sufficient for some biological microfluidic functions. In fact, in **Papers IV** and **V**, xurography was used to produce multi-layered PDMS microfluidic devices with features down to 250 μm in size, for cell culture applications.

Subsequent to production of patterned elastomer, the material is sealed against a flat surface to form the microfluidic channel. In the case of PDMS, it is commonly bonded against another PDMS piece or glass as they are both compatible with plasma bonding techniques (Figure 5). Corona discharge or plasma treatment activates substrate surface groups via conversion and oxidation of terminal methyl groups (Si–CH_3) into surface-bound silanol groups (Si–OH).[125,126] The radical groups between two opposing treated layers can then be brought in contract to catalyse the formation of siloxane covalent bonds (Si–O–Si).[125] This process can be repeated until the full structure is formed, in a layer-by-layer manner. Care has to be taken during the bonding process to ensure optimal alignment between the patterned layers. The surfaces also have to be kept clean and free of particulate matter to ensure ideal bonding. Despite these requirements, plasma bonding of PDMS and glass remain a popular choice for rapid prototyping of chips and the technique was used in **Paper II, III, IV** and **V**.

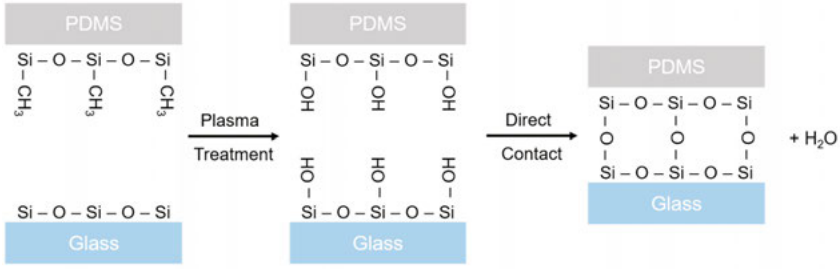

Figure 5. Plasma bonding process for PDMS and Glass substrates after plasma treatment.

3.2.3. Additive Manufacturing for Microfluidics

Additive manufacturing is rapidly becoming more commonplace as a powerful and convenient technique for precision fabrication. There are multiple 3D printing techniques available,[127] but the ones that are most relevant to this thesis are Stereolithography (SLA) and Fused Filament Fabrication (FFF). SLA is similar to photolithography in the sense that a photocurable resin is used and exposed to a high-energy laser that causes a liquid resin to crosslink and solidify at specifically treated voxels, or a layer of voxels at once using the digital light processing (DLP) technique.[127] Voxels are cross-linked in sequence and layer by layer until the full shape of the target part is generated. It is a relatively fast technique; however, SLA prints are not fully biocompatible due to leaching of uncured monomers from the final part and are thus not ideal for direct cell culture without prior treatment.[128,129] Nevertheless, the technique was used in **Paper I** to generate the mould used for soft PDMS lithography.

FFF, on the other hand, involves the production of the structure using a heated nozzle from which a melted plastic filament is extruded.[130] The plastic solidifies on the build plate, which eventually builds out the structure as the nozzle travels along the volume of the target structure.[127] The technique is highly customizable and several parameters can be altered (such as layer height, infill density, nozzle width) leading to changes in print speed and resolution. Different filament materials are available (such as PLA, Nylon and ABS) allowing for prints with different mechanical and chemical properties.[130] The technique was used in **Paper V** to manufacture a modular component of the microfluidic device used to hold the biomaterial in place.

3.2.3. Microfluidics Set-up

Contrary to expectations of miniaturization evoked by the concept of lab-on-chip devices, there usually is a need for associated supportive equipment to ensure the functionality of the chip, thereby expanding the bulk of the total

set-up (Figure 6). The equipment includes pumps, reservoirs, sensors, valves, etc. Such extrinsic factors may limit chip throughput due to formation of process bottlenecks. As such, it is vital to ensure available ancillary capacity prior to the start of the experiment. In addition, if cell culture is to be included into a microfluidic chip, it is critical to establish sterilization routines for both the chip and complimentary equipment prior to cell seeding to avoid contamination. As such, the consideration of what is fully needed to actually run the chip is useful to internalize during the chip design process.

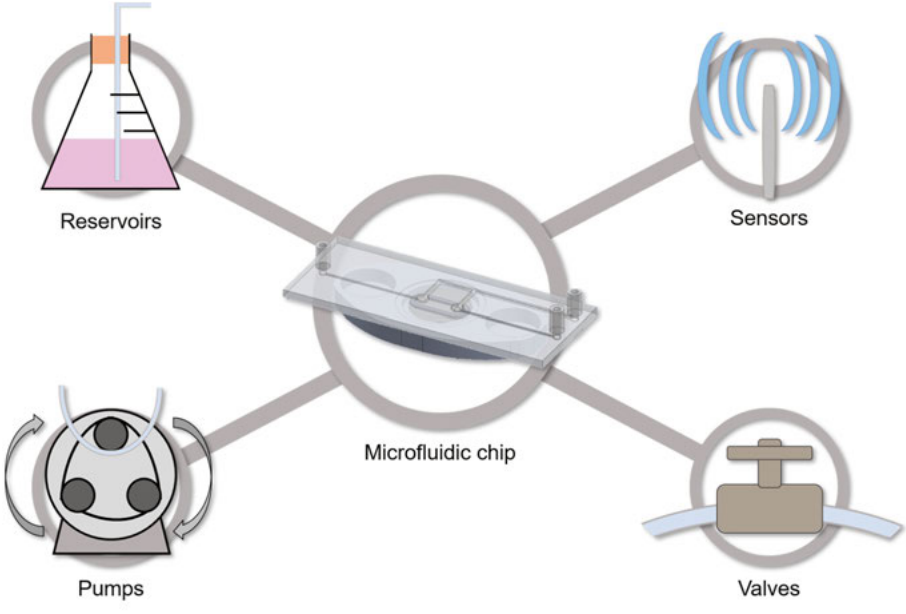

Figure 6. Dependence of microfluidic chip systems on essential supportive devices such as reservoirs and pumps to ensure proper functionality.

Within the chip, flow can be built up through passive (*e.g.* capillary or gravitational) or active (*e.g.* electrophoresis or pressurization) means. Within all included publications in this thesis, active microfluidics (through pressurization) was used to drive the fluid, either through action of a syringe pump or a peristaltic pump. Syringe pumps function by pulling/pushing a loaded syringe using a stepper motor, which generates a specific pulse-free flow that is well suited for the study of the effects of exact flow rates on biological processes. On the other hand, peristaltic pumps work by driving a rotary actuator against flexible tubing, which forces the contained fluid in the tubing to be pumped in the direction of actuation. Unlike syringe pumps, the flow profile is pulsatile and is somewhat reminiscent of the pulsatile nature of physiological blood flow. In the supplementary section of **Paper III**, a minor evaluation into flow dynamics of both perfusion options was performed.

Chapter 4. Summary of Included Publications

4.1 Summary of Paper I

In this work, the interrelations of surface properties, protein adhesion and cell attachment were investigated for calcium-deficient hydroxyapatite (CDHA). Following data collection, the results were fit to a predictive numerical model.

To better understand protein attachment, CDHA was first incubated in a bovine serum albumin (BSA) solution for 3 days, resulting in 50% of BSA solute adsorption on the material. The same CDHA sample was then transferred into BSA-free buffer to assess protein desorption, netting 18% BSA detachment over 3 days. As such, CDHA is surmised to display strong affinity towards BSA binding. Furthermore, CDHA-BSA kinetics were satisfactorily captured by a mathematical model based on the Langmuir isotherm, with modelling errors below 6 % and 12 % for adsorption and desorption, respectively. Such a good fit implies a lack of protein penetration into the material bulk despite CHDA porosity. Next, adhesion of murine MC3T3-E1 pre-osteoblasts over time to CDHA pre-treated with serum-supplemented medium for different periods was investigated. Cell adhesion increased over time for all cases, but the greatest increase was seen for CDHA samples that were not initially pre-incubated with serum. This increase was possibly due to concurrent attachment of proteins and cells in the seeding medium early in the culture, which greatly enhanced the cell adhesion abilities of untreated CHDA. Moving on, a similar computational model for cell adhesion was prepared, which was coupled to the aforementioned protein kinetics model, thus permitting a rough prediction of cell attachment for given CDHA surface protein concentrations. Finally, as an auxiliary assessment, the eccentricity of the attached cells was quantified. Cells cultured on protein-rich CDHA surfaces showed greater eccentricity and spread compared to those on protein-poor surfaces, thus signifying the role of protein as an interface medium for cell-surface interactions.

In conclusion, it was possible to model the relationship between substrate surface properties and resultant protein and cellular attachment dynamics using the Langmuir isotherm. The development of this model may serve as a basis for predictive models of cellular attachment as a function of material surface properties.

4.2 Summary of Paper II

In this publication, the extent of uncured polydimethylsiloxane (PDMS) monomer leaching and the extent of associated effects on *in vitro* culture was investigated. The viability of select treatments of PDMS prior to culture were also assessed for leaching reduction.

Atomic concentrations of silicon ions ([Si]) were measured to represent overall uncured monomer concentrations. Using a single-channel PDMS microfluidic chip, quantification of leached [Si] was performed at flow rates of 0, 0.5, 1.5 and 4.5 µl/min for up to 50 h using culture medium. It was observed that in all perfused conditions, [Si] decreased significantly over time, reaching culture medium control levels more quickly at higher flow rates. In comparison, [Si] release in static samples was at least 3-times higher than flow conditions. Using mass spectrometry, the identity of the majority leached monomer was ascertained to be trimethylsilyl, a fragment of the PDMS polymer chain. These results confirmed the leaching of Si-based monomers and indicated that higher flow rates may constitute one possible strategy for mitigation of high [Si] in culture medium.

Examined strategies for reduction of [Si] release constituted variations to PDMS curing temperatures and times. It was determined that increases in PDMS curing period significantly reduced bulk [Si] release, whereas temperature had minimal effect. Finally, using PDMS-leached culture medium, effects on proliferation and differentiation (via ALP activity) were assessed for murine osteoblasts (MC3T3-E1) and human mesenchymal cells (hMSC) over 14 days. In the case of MC3T3-E1 cells, [Si] leaching had no effect on cell viability but showed signs of ALP activity inhibition at higher [Si] on day 7 of culture. As for hMSCs, there was no significant effect of leached [Si] on proliferation and differentiation processes.

This work indicates that careful consideration needs to be taken during the design and fabrication of PDMS-based microfluidic chips, as leached [Si] may have undesirable effects on some cell types.

4.3 Summary of Paper III

In this publication, the feasibility of microfluidics as a tool for *in vitro* assessment of calcium-deficient hydroxyapatite (CDHA) under more physiological-like conditions was examined.

According to literature, there is a mismatch between *in vivo* and *in vitro* biomaterial assessments, leading to sunken time and cost. CDHA is commonly used as a filling material for bone lesions, but displays substandard biocompatibility *in vitro*, mainly due to a high degree of ion reactivity. Therefore, a CDHA-integrated microfluidic chip was constructed and used to characterize ion reactivity at different flow rates (2, 8 and 14 µl/min). The results indicated an inversely proportional relationship between ionic fluctuation in culture medium and the applied flow rate, thus suggesting that higher flow rates were a valid strategy to shield against material-induced ionic changes. Afterwards, murine MC3T3-EI pre-osteoblasts were cultured on-chip under the same flow rates, as well as on static CDHA samples in a well plate. Cell numbers were counted at 6 and 72 h as an estimate of short-term proliferation, with maximal increases seen at flow rates of 2 and 8 µl/min. Based on these results, a flow of 8 µl/min was selected for further analysis, which consisted of MC3T3-E1 culture on-chip for a longer duration of 10 days and subsequent assessment for LDH and ALP activity corresponding to proliferation and differentiation markers, respectively. Over the 10-day period, sustained proliferation and the formation of a confluent cell layer with high resultant LDH activity was observed on-chip, which was significantly higher than corresponding static samples. As for differentiation, both cells cultured on- and off-chip displayed low ALP activity, indicative of low differentiation behaviour. This may suggest that CDHA under the evaluated conditions of flow promotes cell proliferation, but may not promote differentiation. In conclusion, this work highlighted the importance of selecting the appropriate culture conditions for *in vitro* analysis of biomaterials, due to disparity in cell phenotype dependent on the culture environment.

4.4 Summary of Paper IV

In this paper, calcium-deficient hydroxyapatite (CDHA) samples of contrasting microstructures were evaluated in an on-chip microfluidics platform to evaluate differences in terms of inflammatory responses and drug delivery.

CDHA formulations of varying microstructure were prepared and appropriately named C-CDHA and F-CDHA, respectively. The materials were prepared as discs for use in static conditions, as well as integrated in a microfluidic chip. To begin with, the protein adsorption ability of the two conditions was compared through incubation of a fluorescently-tagged bovine serum albumin (f-BSA). Evaluation of CDHA sample cross-sections demonstrated that f-BSA penetrated most deeply in C-CDHA samples, particularly when integrated on-chip, which is understandable given the larger particle sizes of C-CDHA samples. Furthermore, ionic reactivity was evaluated via direct exposure of CDHA to culture medium and it was observed that on-chip samples displayed increased shielding against ionic changes compared to static conditions. To a lesser extent, C-CDHA samples displayed more ionic shielding compared to F-CDHA. Drug loading of Trolox and release from CDHA substrates was also examined. Release was most pronounced in the first hours of incubation, with on-chip samples demonstrating higher leached Trolox concentrations than static samples. Moving on, murine RAW 264.7 macrophages were used to evaluate CDHA biocompatibility. Proliferation was significantly elevated for cells grown on on-chip compared to cells grown on static discs, which is probably due to better ionic shielding and nutrient delivery under flow. Incidentally, there was no significant difference between C- and F-CDHA in terms of cell growth. Inflammatory response was also investigated via quantification of tumour necrosis factor alpha (TNF-α) release from cultured cells on CDHA. Interestingly, there was no significant difference in TNF-α release between on-chip and static samples.

In conclusion, the CDHA-on-chip platform demonstrated applicability for the assessment of multiple relevant parameters (such as protein adsorption, drug release and inflammation response) under flow conditions. Similarly, as in paper II, this work illustrated the sensitivity of cellular response towards *in vitro* culture condition changes and the resultant need for careful consideration of optimal conditions prior to the biomaterial evaluation process.

4.5 Summary of Paper V

A universal biomaterial on-chip platform (UBoC) was developed to allow for fair and standardized comparisons of biomaterials, with included modularity to allow for a wide range of analyses. Using the device, it was possible to integrate materials of varying properties, sizes and shapes and perfuse culture medium along the substrate surface.

The UBoC device was constructed using a combination of techniques including 3D-printing, soft lithography and laser etching. The culture area was defined through a re-sealable gasket and was identical in area to that of a 96-well plate. An in-line bubble trap was included on-board the chip and helped to increase culture robustness and minimize bubble-induced damage. Calcium-deficient hydroxyapatite (CDHA), titanium alloy (Ti) and fibrin hydrogels were integrated into the device and tested for biocompatibility by culturing murine L929 fibroblasts for up to 5 days on the material surfaces under a constant 2 µl/min flow. Successful proliferation was observed for all substrates, with CDHA and fibrin showing the highest cell counts, followed by Ti. Furthermore, mechanosensitivity evaluations were demonstrated through culture of murine MC3T3-E1 pre-osteoblasts on a Ti substrate on-chip with subsequent exposure to a range of shear stimulations over 2 min, using Ca^{2+}-sensitive dyes for quantification. As flow rate was increased, cells displayed progressively higher calcium flux, with 50 µl/min triggering the highest level of flux, while increased flow rates beyond 50 µl/min reaching a signal plateau. Finally, co-culture was also verified as an available means of analysis after slight modification of the UBoC device. L929 and MC3T3-E1 cells were seeded abreast in the chip using laminar flow dynamics, with each cell type nourished with its respective optimum medium. Concurrent proliferation of both cell types was observed and clear separation was maintained for up to 3 days of culture.

In conclusion, the UBoC device has potential for use as a modular tool to augment *in vitro* biomaterial screening pipelines by making flow-based studies (such as mechanotransduction and co-culture) more accessible regardless of the physical features of the sample.

Final Thoughts and Future Perspectives

The end goal of clinical testing is to ensure the optimal performance of biomaterials when implanted into patients, but due to financial and safety concerns, a level of generalization is first needed. This led to consideration of animal testing (termed "*in vivo*") as the gold standard, with the specific species being chosen dependent, for example, on the size of the implant and relevant parameters. Despite significant genetic variation compared to humans, animals provide valuable information due to the possession of intact 3D tissue architecture, with corresponding vasculature and immune systems that emulate the human healing process. Regardless, success in such investigations does not guarantee suitability in humans, with further rounds of clinical human testing needed before complete endorsement can be granted. Indications about the relative success of the implant can nevertheless be garnered from *in vivo* testing before moving towards more complex, costly and risky human validation.

However, as biomaterial research output increased over the last decades, *in vivo* trials have become capacity constrained, more expensive and challenged by ethical concerns, thereby necessitating an additional level of generalization which took the form of *in vitro* testing. Such evaluations assay the performance of material-laden cell cultures for parameters such as biocompatibility and immunogenicity. While *in vitro* testing can give some predictions about the expected responses of the material *in vivo*, the vast difference in culture environment is likely to bias cellular responses away from what is considered physiologically-relevant. As such, my main vision throughout this PhD dissertation was the development of solutions that may ameliorate this particular lack of consistency between *in vitro* and *in vivo* trials.

One method of improving the information output of *in vitro* trials is the incorporation of *in silico* analytic pipelines, whereby computational models are utilized to capture cellular responses to different stimuli. Such approaches have found popular use in fields such as drug discovery and protein science due to high-throughput screening capabilities of the technique. In a similar vein of thinking as before, *in silico* analytics can be seen as a generalization of the *in vitro* culture environment, where the system is mathematically simplified to generate an output based on inputted biological parameters. For example, in

Paper I, it was possible to breakdown and explain the attachment dynamics of protein and cells on hydroxyapatite (HA) surfaces in terms of a simple formula based on the Langmuir isotherm. In theory, with such a formula, it was possible to generate a prediction of cell attachment for a given protein concentration. However, in practice, *in silico* models may be unable to provide a complete predictive description of a biological process due to inherent complexities and knowledge gaps about the given process. In addition, processing power limitations may also make it infeasible to model the entire physiological system in an assumption-free manner. Nevertheless, as our knowledge base and computational power continues to grow, it is expected that *in silico* analysis will find greater usage within the field of biomaterial science, particularly for screening applications.

Alternatively, microfluidics forms the basis of one strategy to achieve the aforementioned vision, where the goal was to innovate upon standard *in vitro* culture methods and add layers of complexity that may bring it closer in line to the conditions experienced by the implant *in vivo.* Microfluidics enables the addition of features such as miniaturization, nutrient perfusion and shear stress, all of which are a step closer to the physiological situation. A wide variety of materials are available for fabrication of microfluidic devices, but care needs to be taken to first understand any potential effects such materials may have, particularly on cell culture. For instance, in **Paper II**, the effects of polydimethylsiloxane (PDMS) were verified for cell viability prior for use in chip fabrication. Nevertheless, looking at the biomaterial on-chip systems developed in **Papers III and IV**, cellular responses appeared deviated from those obtained on static well plate culture, but were overall trending in a positive direction, showing high levels of cell attachment and proliferation. However, such trends do not necessarily confirm the superiority of biomaterial-on-chip systems against standard culture formats, rather that the focus is on the dissimilar cell behaviour garnered under different conditions of evaluation. As such, there is a need for careful selection of appropriate experimental settings beforehand to ensure ideal relevance for the biomaterial and its intended applications.

As biomaterial on-chip systems continue to evolve in terms of research activity, there may eventually be a growing need for a base level of standardization between the different platforms to enable a fair degree of comparison. By comparisons, what was meant was exposure of biomaterial formulations to precisely the same experimental conditions. Likewise, usage of the device by others in multi-centre studies should net the same result assuming all conditions are kept identical. The devices should also be designed to enable accessibility and ease of use by labs with limited microfabrication experience. To achieve this level of consistency, the Universal Biomaterial on-chip was designed (as described in **Paper V**) with biomaterial flexibility, device

modularity and user accessibility in mind. The vision with this device was to be able to incorporate materials of different shapes and properties and evaluate them under the same conditions. This was successfully achieved with the subset of biomaterials tested and future work would focus on further validation of the device using additional cell types and relevant biomaterial substrates. In addition, the modularity of the device can be further expanded upon to for example include support and interfaces for integrated microsensors.

While the strategies of *in silico* analysis and microfluidics displayed potential in enhancing the informativity of pre-*in vivo* testing phases, it is still of critical importance to validate and benchmark all the developed systems so far against clinical data to confirm a satisfactory degree of correlation to the current gold standard. This process of comparison can also be used as a form of ongoing audit of device development to ensure steady progress and identify milestones whereby the on-chip assessment strategy can be said to have achieved the necessary degree of physiological relevance to guarantee consistency to *in vivo* results. In future related work, this form of continual review would constitute a major part of future analysis that will shape any adjustments or alterations to be made to our developed techniques.

In summary, this thesis outlined a case for the integration of microfluidics into existing *in vitro* procedures as an attempt to increase relevance to *in vivo* testing and thus improve predictive power. There were indications that such an approach did lead to differences in cellular responses compared to standard static conditions, but validation against *in vivo* models is still required before conclusions can be fully drawn about the efficacy of microfluidics in this context. The same can be said about *in silico* analytics, which showed promise in generalization of *in vitro* data, but its applicability towards other assays needs to be established before it can be confirmed as a suitable screening tool for biomaterial development. All in all, this dissertation can be distilled into a message which stresses the importance of setting appropriate *in vitro* assessment conditions most ideally suited for the targeted and expected *in vivo* implant context.

Popular Summary

Due to advances in medicine, people generally live longer and reach older ages. The elderly are generally more liable towards injury during daily life, resulting in a greater incidence in issues such as musculoskeletal defects or bone fractures. In addition, the body is less able to intrinsically repair itself at an advanced age, making medical intervention more critical towards the healing process.

In the case of bone, when faced with a defect or diseased area, the best approach is to extract bone from another area from the same patient and implant it into the affected area. Using material from the same body would be most accepted by the affected individual's immune cells and contains all the physiological cues necessary to support the healing process. However, such a procedure would necessitate double surgeries, which would put additional burden on the patient, as well as the amount of bone available for use is limited. Alternatively, bone can be extracted from other patients, but this is also limited in quantity and may induce detrimental immune reactions in the treated patient.

One solution is to use biomaterials, which are materials originating from nature or synthetic processes that can substitute for physiological tissue and bring about a supportive or regenerative outcome in the body. Nowadays, biomaterials are extensively used in clinics, with some examples being tooth implants (such as screws and crown implants), sutures for wound closure and contact lenses. As these materials are in direct contact with the body, it is important to ensure that they are inert and induce no harmful bodily interactions. Over the past decade, biomaterials have gotten more sophisticated and able to directly promote healing in the tissue by releasing helpful drugs or being more easily processed by the body. However, despite the push for further biomaterial development, few such innovations have reached the market. This is due to rigorous testing procedures on the lab bench, in animals and in humans that are important to certify the material's safety and performance.

In the first steps of validation, live cells are placed on the material and cultured outside of the body in specialized chambers and liquid media that keep the cells alive. The reaction of the cells to the material is then investigated using

techniques such as microscopy or chemical analysis. These reactions give insight about the possible implications of biomaterial-cell interactions. For example, if the cells put on the material are seen to not grow well or die, then this might mean the material is toxic in some way and will not be allowed to proceed to later stages of testing until the issue is rectified. While such analysis is easy and cheap, there has been some criticism of its ability to fully depict the physiological situation, as it is an exceptionally simple representation of the complex body situation. As such, results obtained from this kind of testing may not correspond well to results obtained with the same material when implanted in animals.

An improvement in lab cell testing methods may be brought about through use of microfluidics, which is a technique that allows for control of small liquid volumes in a precise way. Microfluidic systems can be made up of a wide variety of materials and are miniature in size, sometimes down to the size of a coin. A defining feature of these systems is that at least one of their dimensions is in the microscale range, which roughly corresponds to the size of a human hair. Physiologically, these systems have the potential to simulate the functionality seen in blood systems in the body, where they are able to deliver nutrients to and remove waste from the cells in a continuous manner. The cells also grow in small compartments and can be placed in close proximity to other cells in an ordered way, which is similar to the tissue situation. Finally, due to precise microfluidic control, drugs or stimulants can be delivered directly to the cells and cell response products can be collected in real-time for analysis of cell behaviour.

Within this thesis, the integration of microfluidic design principles for the evaluation of biomaterials was investigated, particularly under conditions that better mimic what is experienced in the body. Parameters I looked into include cell growth and differentiation, but also characterization of the materials with parameters such as protein adhesion, drug release and leaching of ions.

In **Paper I**, the focus was on the characterization of biomimetic hydroxyapatite (otherwise known as CDHA) in terms of its protein attachment ability and how well it is able to support cell attachment in static conditions. This is relevant as cells are normally unable to directly interact with the material surface, but instead interact via a proteinaceous layer that builds up on the material directly after implantation and contact with blood and tissue fluid. In this paper, I also investigated if it was possible to construct a mathematical equation which could capture the biological behaviour in a predictable manner.

As for **Paper II**, the focus was on the verification of a silicon-based polymer (known as PDMS), commonly used for microfluidics, for its applicability for use with cells without harming them. Specifically, the ability of PDMS to

leach silicon ions was investigated, as these ions were reported to influence bone cell activity. This was done through measurement of the concentration of released ions and comparison between flow and static conditions. Interestingly, through variation of PDMS curing conditions, a reduction in silicon ion leaching was seen. Regardless, it was demonstrated that PDMS still had a measurable effect on phenotypic expression of certain cell strains, which implies that care needs to be taken when selecting the cell type for use in PDMS devices.

After verification of PDMS as a suitable substrate for microfluidic chip fabrication, in **Paper III**, CDHA was integrated into an on-chip device. CDHA is known to have a high ionic reactivity, whereby it readily uptakes calcium and releases phosphate from a solution it is immersed in. It is believed that in the body, the ionic reactivity is compensated for by the action of the circulatory system, thus allowing for the implant to be integrated more successfully. However, in static testing conditions on the bench, the ionic reactivity quickly alters the medium composition and inhibits cell culture. However, when placed on-chip under continuous flow, the ionic effects are shielded against and ideal medium conditions are maintained. A large difference was also seen for cell culture, where cells cultured on-chip showed a much higher degree of growth compared to cells grown on the material with no flow. As such, it can be determined that application of perfusion promotes cell survival on CDHA and may constitute a better analytical laboratory platform for biomaterial evaluation, specifically for CDHA and the cells tested.

Moving on, in **Paper IV**, the CDHA-on-chip device was further expanded upon to determine its applicability at answering more sophisticated biomaterial-related research questions. CDHA samples of differing microstructures were prepared and investigated for differences in the protein penetration dynamics and any associated effects of flow, with results indicating that formulations prepared with coarser and larger particle sizes displayed deeper infiltration by the protein, particularly under flow. Likewise, drug-loaded CDHA samples were analysed in a similar manner under flow, with flow-exposed samples showing a greater degree of drug release compared to static samples. Finally, immune cells were seeded on the CDHA samples on-chip and grown over 3 days. Results indicated that perfused samples showed greater cell growth, mirroring the results obtained in **Paper III.** Overall, it was possible to use the system to physically characterize biomaterials and study their immunogenicity, thus highlighting the applicability of the technique.

Finally, in **Paper V**, a microfluidic device, known as the Universal Biomaterials-on-Chip (UBoC), capable of integrating a wide variety of biomaterials (including cements, metals and hydrogels) was constructed, thereby enabling fair comparison between materials of different properties. The device was also

modular and could support additional study functionalities for more extensive biomaterial evaluation. In addition, a bubble trap was included to remove bubbles in the media, which would otherwise damage and kill the cells if they come in contact. Overall, using the device, cells proliferated successfully on CDHA (porous cement), titanium (hard metal) and fibrin (hydrogel) over 5 days. As an example of further functionality, the UBoC device was used to investigate the sensitivity of cells to flow by applying gradually increasing flow magnitudes. It was observed that as flow rate was increased, there was a greater increase in cell response intensity. Finally, by slightly modifying device components, it was possible to grow two different cell types on the same substrate in the same chip, thus allowing their interactions to be studied.

Through this thesis, the potential for integration of microfluidics in biomaterial lab testing workflows was demonstrated. The large differences observed between on-chip and static studies stressed the importance of selecting appropriate culture conditions before the start of material analysis. Despite this, due to the vastly complex situation present in the body, further work and tuning of devices is needed before we can claim a true representative culture environment.

Populärvetenskaplig Sammanfattning

Tack vare de medicinska framsteg som gjorts lever människor i dag längre och når högre åldrar. Äldre är generellt mer utsatta för skador vilket också resulterar i att problem med muskelskador och benbrott också ökar. Dessutom är kroppen mindre förmögen att reparera sig själv i en hög ålder, vilket kräver medicinsk ingrepp i större grad.

När det gäller läkning av större benskador är den bästa metoden att extrahera ben från ett annat område från samma patient och implantera det i det skadade området eftersom patientspecifikt ben lättare accepteras av immunsystemet och innehåller alla fysiologiska signalämnen som är nödvändiga för att stödja läkningsprocessen. En sådan procedur kräver dock dubbla operationer, vilket lägger ytterligare börda på patienten. Alternativt kan ben extraheras från andra patienter, men detta är också begränsat i kvantitet och kan inducera skadliga immunreaktioner hos den behandlade patienten.

En lösning är att använda biomaterial, som är material som härrör från naturen eller syntetiska processer och som kan ersätta fysiologisk vävnad och stödja kroppen under läkningen. Nuförtiden används biomaterial flitigt på kliniker, där några exempel är tandimplantat (t.ex. skruvar och kronimplantat), suturer för sårtillslutning och kontaktlinser. Eftersom dessa material är i direkt kontakt med kroppen är det viktigt att se till att de är inerta och inte framkallar alltför stora negativa biverkningar. Under det senaste decenniets forskning har nya sofistikerade biomaterial tagits fram som inte bara stödjer läkningsprocessen men också kan påskynda den genom att släppa ut läkemedel. Men trots det ökade behovet av nya biomaterial inom vården har få sådana innovationer nått klinikerna. Detta beror till stor del på de rigorösa testprocedurer på laboratoriebänken, på djur och på människor som är viktiga för att certifiera materialets säkerhet och prestanda.

I de första stegen av valideringen placeras levande celler på materialet och odlas utanför kroppen i specialiserade kammare med flytande media som håller cellerna vid liv. Cellernas reaktion på materialet undersöks sedan med hjälp av tekniker som mikroskopi eller kemisk analys. Dessa reaktioner ger insikt om de möjliga konsekvenserna samspelet mellan cellerna och biomaterialet kan leda till. Om man till exempel ser att cellerna som sätts på materialet inte

växer bra eller t.o.m. dör, kan det betyda att materialet är giftigt på något sätt och det tillåts därför inte fortsätta till senare teststadier förrän problemet är åtgärdat. Även om en sådan analys är enkel och billig, har det förekommit en del kritik mot dess oförmåga att fullständigt avbilda den fysiologiska situationen, eftersom det är en exceptionellt enkel representation av den komplexa kroppssituationen. Det är därför inte säkert att resultaten som erhållits från denna typ av tester motsvarar resultat som erhålls med samma material när de implanteras i djur.

En förbättring av de cellbaserade testmetoder som används på laboratoriet kan åstadkommas genom användning av mikrofluidik, vilket är en teknik som möjliggör kontroll av små vätskevolymer på ett exakt sätt. Mikrofluidala system kan tillverkas i en mängd olika material och är i miniatyrstorlek, ibland ner till storleken av ett mynt. En utmärkande egenskap hos dessa system är att åtminstone en av deras dimensioner är på mikrometer skalan, vilket ungefär motsvarar storleken på ett människohår. Fysiologiskt har dessa system potential att simulera den funktionalitet som ses i blodsystemet i kroppen, där de kan leverera näringsämnen till och ta bort avfall från cellerna på ett kontinuerligt sätt. Cellerna växer också i små fack och kan placeras i nära anslutning till andra celler på ett ordnat sätt som liknar hur mänsklig vävnad är uppbyggd. Slutligen, på grund av den precisa kontroll som mikrofluidala system erbjuder kan läkemedel levereras direkt till cellerna och deras respons snabbt samlas in i realtid för analys av cellbeteende.

I denna avhandling har integrationen av mikrofluidala designprinciper för utvärdering av biomaterial, särskilt under förhållanden som bättre efterliknar det som upplevs i kroppen, undersökts. Parametrar jag har tittat på inkluderar celltillväxt och differentiering, samt karakterisering av materialen med parametrar som proteinadhesion, läkemedelsfrisättning och urlakning av joner.

I **Paper** I låg fokus på karakteriseringen av biomimetisk hydroxyap-atit (mer känt som CDHA) när det gäller dess förmåga att binda proteiner och hur väl materialet kan stödja cellvidhäftning under statiska förhållanden. Detta är relevant då celler normalt inte kan interagera direkt med materialytan utan istället interagerar via ett proteinhaltigt lager som byggs upp på materialet direkt efter implantation och kontakt med blod och vävnadsvätska. I denna artikel har vi också undersökt om det var möjligt att konstruera en matematisk ekvation som kunde fånga det biologiska beteendet på ett förutsägbart sätt.

När det gäller **Paper II** låg fokus på verifieringen av en kiselbaserad polymer (känd som PDMS), vanligen använd för mikrofluidik, för användning med celler utan att skada dem. Specifikt undersöktes urlakning av kiseljoner från PDMS eftersom dessa joner tidigare har rapporterats påverka bencellsaktivitet. Detta gjordes genom mätning av koncentrationen av frigjorda joner och

jämförelse mellan flödande och statiska förhållanden. Intressant nog sågs en minskning av kiseljonläckaget från materialet när härdningsprocessen ändrades. Oavsett vilket visade det sig dock att PDMS fortfarande hade en mätbar effekt på det fenotypiska uttrycket hos vissa cellstammar, vilket innebär att försiktighet måste iakttas när man väljer celltyp för användning i system tillverkade av PDMS.

Efter verifiering av PDMS som ett lämpligt substrat för tillverkning av mikrofluidala chip, integrerades CDHA i en on-chip-enhet i **Paper III**. CDHA är känt för att ha en hög jonisk reaktivitet, varvid den lätt tar upp kalcium och frigör fosfat från den lösning den är nedsänkt i. Man tror att den joniska reaktiviteten i kroppen kompenseras av cirkulationssystemets inverkan, vilket gör det möjligt för implantatet att ändå framgångsrikt integreras med benvävnaden men i statiska testförhållanden i laboratoriet förändrar den joniska reaktiviteten snabbt mediets sammansättning och hämmar celltillväxt. I det här arbetet visade vi att när CDHA placeras i ett mikrofluidalt system under kontinuerligt flöde upprätthålls idealiska mediumförhållanden även om materialet kan påverka jonbalansen. En stor skillnad sågs även för cellodling, där celler odlade på chip visade en mycket högre grad av tillväxt jämfört med celler odlade på materialet utan flöde. I och med detta kan det fastställas att applicering av flöde främjar cellöverlevnad på CDHA och kan utgöra en bättre analytisk laboratorieplattform för utvärdering av biomaterial, specifikt för CDHA och de celler som användes i den här studien.

I **Paper IV**, utökades CDHA-on-chip-enheten ytterligare för att bestämma dess tillämpbarhet för att svara på mer sofistikerade biomaterialrelaterade forskningsfrågor. CDHA-prover av olika mikrostrukturer bereddes och undersöktes för skillnader i proteinpenetrationsdynamik och eventuella associerade effekter av flöde, med resultat som indikerar att formuleringar framställda med grövre och större partikelstorlekar visade djupare infiltration av proteinet, särskilt under flöde. Likaså analyserades läkemedelsladdade CDHA-prover på liknande sätt under flöde, där flödesexponerade prover visade en högre grad av läkemedelsfrisättning jämfört med prover i statiska miljöer. Slutligen odlades immunceller på CDHA-proverna på chipet under 3 dagar. Resultaten visade att prover som utsattes för ett flöde av cellodlingsmedia visade högre celltillväxt, vilket speglade resultaten från **Paper III**. Sammantaget var det möjligt att använda systemet för att fysiskt karakterisera biomaterial och studera deras immunogenicitet, vilket belyser teknikens tillämpbarhet.

Slutligen, i **Paper V**, konstruerades en mikrofluidalt system kallat Universal Bio-materials-on-Chip (UBoC), som kan integrera en mängd olika biomaterial (inklusive cement, metaller och hydrogeler), vilket möjliggör rättvis jämförelse mellan material med olika egenskaper. Enheten är modulär och kunde stödja olika typer av analyser för en mer omfattande utvärdering av

biomaterial. Dessutom ingick en bubbelfälla för att ta bort bubblor i mediet, som annars skulle skada och döda cellerna om de kommer i kontakt. Med hjälp av enheten odlades celler framgångsrikt på CDHA (porös cement), titan (hårdmetall) och fibrin (hydrogel) under 5 dagar. Som ett exempel på ytterligare funktionalitet användes UBoC-enheten för att undersöka cellers känslighet för flöde genom att applicera gradvis ökande. Det observerades att när flödeshastigheten ökades, var det en större ökning av cellresponsintensitet. Slutligen, genom att modifiera enhetskomponenter var det möjligt att odla två olika celltyper på samma substrat i samma chip, vilket gjorde det möjligt att studera deras interaktioner.

Genom denna avhandling har potentialen för integrering av mikrofluidala system i de laboratoriebaserade testningsarbetsflödena för biomaterial demonstrerats. De stora skillnaderna som observerades mellan on-chip och statiska studier betonar vikten av att välja lämpliga odlingsförhållanden innan materialanalysen påbörjas. Trots detta, på grund av den oerhört komplexa situationen i din kropp, krävs ytterligare arbete och justering av enheter innan vi kan göra anspråk på en verklig representativ odlingsmiljö för testning och utveckling av nya biomaterial.

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