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Applications of Additive Manufacturing for Advancing Cell Models from 2D to 3D

CHRISTINA STELZL



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Abstract

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Two-dimensional (2D) cell culture systems are widely used in preclinical research due to their ease of handling and standardisation, but do not adequately reflect key aspects of the complex three-dimensional (3D) physiological microenvironment. This limits the predictive value of *in vitro* studies for both drug development and biomaterials research. The overall aim of this thesis was to explore how additive manufacturing supports the transition from 2D to more advanced 3D cell culture models.

In Study I, CombiCTx, a cell culture device for combinatorial anti-cancer drug testing, was developed. The system enables the formation of overlapping drug gradients through diffusion in a hydrogel matrix, and an assay and imaging analysis protocol was established. Using breast cancer cells, it was demonstrated that the assay can identify synergistic drug effects and that, for the drugs tested, these effects were spatially confined to specific regions of the assay space, highlighting the importance of diffusion processes not captured in standard 2D assays.

In Study II, an open source extrusion-based bioprinter based on the E3D motion system was established to increase accessibility to bioprinting technologies. The system supports multimaterial printing and FRESH bioprinting. Collagen scaffolds and cell-laden laminin-containing constructs were printed, and high cell viability was maintained, demonstrating the suitability of the platform for generating 3D cell culture environments.

Studies III and IV focused on biomaterials for bone regeneration. In Study III, the biosafety of a phosphoserine (pSER)-modified calcium phosphate bone adhesive was evaluated. Both *in vitro* and *in vivo* results indicated good biocompatibility, with no evidence of adverse immune reactions or ectopic bone formation.

In Study IV, 3D bioprinted collagen-silica hybrid scaffolds modified with pSER were investigated. *In vitro* experiments showed a dose-dependent effect of pSER in combination with calcium phosphate on cell viability. *In vivo*, mineralised scaffolds promoted bone formation, suggesting an osteogenic potential of these materials.

In conclusion, the studies presented in this thesis demonstrate that additive manufacturing can be used to develop more advanced *in vitro* models and to investigate biomaterials in controlled 3D environments. These approaches will contribute to improving the translation of preclinical findings into clinical applications.

Keywords: 3D printing, additive manufacturing, 3D bioprinting, *in vitro*, biomaterials, combinatorial drug screening, bioink

Christina Stelzl, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden.

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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. Stelzl, C., Lerma-Clavero, A., Camenisch, S., Simon, B., Eriksson, O., Degerstedt, O., Lennernäs, H., Heindryckx, F., Kreuger, J., O'Callaghan, P. (2026) CombiCTx: screening diffusion gradients of anti-cancer drug combinations. *Lab on a Chip*. 695-710
- II. Engberg, A., Stelzl, C., Eriksson, O., O'Callaghan, P., Kreuger, J. (2021) An open source extrusion bioprinter based on the E3D motion system and tool changer to enable FRESH and multimaterial bioprinting. *Scientific Reports*, 11(1):21547
- III. Hulsart-Billström, G., Stelzl, C., Procter, P., Pujari-Palmer, M., Insley, G., Engqvist, H., Larsson, S. (2020) In vivo safety assessment of a bio-inspired bone adhesive. *Journal of Materials Science: Materials in Medicine*, 31(2):24
- IV. Stelzl, C., Lundqvist, M., Norein, N., Sehic, E., Carlsson, E., Procter, P., Hilborn, J., Hulsart-Billström, G. (2026) Phosphoserine-enriched dense collagen bioink. *Manuscript*

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Publications not included in the thesis:

- I. O'Callaghan, P., Engberg, A., Eriksson, O., Fatsis-Kavalopoulos, N., Stelzl, C., Sanchez, G., Idevall-Hagren, O., Kreuger, J. (2022) Piezo1 activation attenuates thrombin-induced blebbing in breast cancer cells. *Journal of Cell Science* 135(7):jcs258809.
- II. Kontakis, MG; Moulin, M; Andersson, B; Norein, N; Samanta, A; Stelzl, C; Engberg, A; Diez-Escudero, A; Kreuger, J; Hailer, NP. (2025) Trabecular-bone mimicking osteoconductive collagen scaffolds: an optimized 3D printing approach using freeform reversible embedding of suspended hydrogels. *3D printing in medicine* 1(1):11

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
α -TCP	alpha-tricalcium phosphate
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
BMU	Basic multicellular unit
CAD	Computer-aided design
Col1a1	Collagen type I alpha 1 chain
CPC	Calcium phosphate cement
DCSi	Dense collagen-silica hybrid bioink
DMEM	Dulbecco's Modified Eagle Medium
DOX	Doxorubicin
EBB	Extrusion based bioprinting
ECM	Extracellular matrix
FDA	Fluorescein diacetate
FBS	Fetal bovine serum
FDM	Fused deposition modelling
FRESH	Freeform reversible embedding of suspended hydrogels
GAG	Glycosaminoglycan
GelMA	Gelatin methacryloyl
HA	Hydroxyapatite
hBMSC	Human bone marrow stromal cell
IL1b	Interleukin-1 beta
IL6	Interleukin-6
LMPA	Low melting point agarose
MC3T3-E1	Mouse preosteoblast cell line
MDCSi	Mineralised dense collagen-silica
MEM	Minimum Essential Medium
MSC	Mesenchymal stem cell
nHA	Nanohydroxyapatite
PBS	Phosphate-buffered saline
PenStrep	Penicillin-streptomycin
PI	Propidium iodide

PLA	Polylactic acid
PM-CPC	Phosphoserine-modified calcium phosphate cement
pSER	Phosphoserine
qPCR	Quantitative polymerase chain reaction
rMSC	Rat mesenchymal stem cell
ROI	Region of interest
RUNX2	Runt-related transcription factor 2
SLA	Stereolithography
SLS	Selective laser sintering
SOX9	SRY-box transcription factor 9
TNF	Tumour necrosis factor
TME	Tumour microenvironment
TRITC	Tetramethylrhodamine-5-isothiocyanate
UV	Ultraviolet
μCT	Micro-computed tomography

Introduction

Over the past seven decades, standard cell culture has been instrumental in facilitating biological and medical discoveries. Two-dimensional (2D) cell models remain widely used to investigate novel biomaterials and drugs *in vitro* and enable the study of complex cellular responses in a simplified and controllable environment. Additionally, their ease of handling and relative cost-effectiveness make them attractive experimental systems. However, 2D cell models fail to fully recapitulate the complex three-dimensional (3D) environment to which cells are exposed to and the physiological processes of human tissues. This limits the translation of preclinical *in vitro* findings into *in vivo* studies and ultimately into medical applications that benefit patients. It is estimated that only a very small fraction (<1%) of drug candidates identified in basic research receive Food and Drug Administration (FDA) approval, and only approximately 5% of oncological drugs entering Phase I clinical trials are successfully approved¹⁻³. Similarly, there is often poor correlation between *in vitro* and *in vivo* results in preclinical biomaterials testing⁴. Beyond the substantial investment of time and resources, this limited translation also raises ethical concerns with regard to the principles of replacement, reduction, and refinement (3Rs) first proposed by Russell and Burch in 1959⁵.

Therefore, advanced cell culture models are needed to bridge the gap between *in vitro* and *in vivo* studies and to enable the modelling of key physiological aspects of human tissues. Advanced models that include cell–cell or cell–extracellular matrix (ECM) interactions, establish physiologically relevant gradients, or allow interactions between multiple cell types are important for improving the predictive value of *in vitro* models and reducing the number of animals used in preclinical research.

Additive manufacturing offers new opportunities to increase the structural and biological complexity of *in vitro* cell models. Conventional 3D printing of thermoplastics or resins enables rapid prototyping and facilitates the production of customisable devices for cell culture. 3D bioprinting of biological materials, such as protein-based hydrogels and living cells, allows the generation of tissue models or biomaterial scaffolds with improved spatial control and

reproducibility. In this thesis, both approaches are used to explore how additive manufacturing can be used in the transition from 2D to 3D cell culture and to create cell culture models for drug research and to study biomaterials for bone tissue engineering.

Background

Why do we want to move from 2D to 3D?

ECM and its implications for cell signalling

The extracellular matrix is a dynamic and complex network of proteins and other macromolecules that provides structural support to cells and tissues and plays a central role in signalling and tissue homeostasis. The main components include collagens, glycosaminoglycans (GAGs), proteoglycans, and a wide variety of non-collagenous ECM proteins^{6,7}. The human core matrisome, the set of ECM proteins comprising collagens, proteoglycans, and ECM glycoproteins, contains approximately 300 proteins⁷⁻⁹, underscoring the complex nature of the ECM. The ECM can be divided into two types, the basement membrane, a thin dense sheet of ECM that cells anchor to, and the interstitial ECM, a more porous mesh-like network, which forms the major part of connective tissues^{7,10}. The ECM composition differs between these two types and is also highly tissue-specific, for example, the mineralised ECM in bone. Additionally, the ECM is highly dynamic and is constantly remodelled through reciprocal signalling between cells and their surrounding ECM to adapt mechanical properties and biochemical cues as necessary⁷. This “dynamic reciprocity” has been recognised since the 1980s¹¹.

Since these ECM–cell interactions are implicated in a vast variety of different tissue functions, it is therefore relevant to include ECM molecules in advanced *in vitro* models. Some examples of commonly used ECM molecules in tissue engineering and 3D *in vitro* models are hyaluronan, fibronectin, laminins, and collagens.

Hyaluronan is a GAG implicated in water retention and plays pivotal roles in development, regeneration, and tissue repair^{6,12}.

Fibronectin is a large multidomain ECM protein that interacts with many other ECM components and cells. It regulates cell adhesion, migration and differentiation, but also mechanical properties of the matrix, and is also found in the basement membrane^{6,13-15}.

Laminins are heterotrimeric adhesion proteins composed of one α -, β - and γ -chain that assemble into a central triple-helical coiled-coil domain. Laminins interact with other components of the ECM, such as collagens, and with cell-surface receptors such as integrins, to form a highly organised network. Through interactions with cell-surface receptors, laminins direct essential cellular behaviours, including adhesion, migration, polarity, differentiation, and survival. Their expression is highly tissue-specific and they are characteristically found in the basement membrane ^{6,13,16}.

Collagens, particularly fibrillar collagens, are among the most abundant proteins in the mammalian body. In mice 12-17% of the total protein content is attributed to collagen with large variations between organs ¹⁷. Each tissue has a specific collagen signature but collagen type I is the most abundant overall ¹⁸. In total, 28 different types of collagens have been identified, all of which are hetero- or homotrimers composed of three polypeptide chains, called α -chains, assembled into a triple helix ^{7,13,19,20}. Some collagens, e.g., collagen I, II and III, and several more, assemble into fibrils through intra- and extracellular post-translational processes. The final collagen fibrils confer mechanical stability to tissues and interact with cells through different receptors, directing, among other processes, cell differentiation and migration ^{13,19,21}.

Integrins are one of the most important families of cell adhesion proteins involved in cell-ECM interactions. These receptors span the plasma membrane and link cells to components of the ECM. Structurally, integrins are heterodimers consisting of α and β subunits, which form at least 24 known combinations. Their short cytoplasmic domains are linked to the cytoskeleton via adaptor molecules ^{6,22}. Through bidirectional signalling, so-called inside-out and outside-in signalling, integrins can not only interact with cells but also sense ECM forces, rigidity and topography, acting as mechanosensors. Thus, they play an important role in different cell behaviours, including but not limited to, cell death and survival regulation, cytoskeleton dynamics, as well as cell migration ^{6,22,23}. The different integrins can bind to various adhesion sites on ECM molecules, for instance RGD motifs present in fibronectin or GFOGER sequences in fibrillar collagens ^{6,19}.

Given the complex nature of ECM-cell interactions, it is unsurprising that changes and dysregulation of ECM composition are implicated in different diseases. In cancer, the matrix undergoes extensive remodelling and this dysregulation may be considered a hallmark of solid tumours ¹⁵. Modifications in the biochemical and biophysical properties of the ECM, including changes

in pore sizes, molecular composition, and stiffness, affect the diffusion within the tumour microenvironment (TME) and thus the distribution of growth factors and other signalling molecules. These changes have widespread implications for metastasis, chemotherapy resistance, and thus, treatment options^{15,24}.

Overall, ECM composition has strong implications for cell behaviour in healthy and diseased tissues *in vivo* and should therefore also be considered in the context of *in vitro* models.

Advantages and limitations of classic 2D cell culture

The beginnings of cell culture as we know today can be traced back to the late 19th century. The first tissue culture protocols were established in the early 20th century, for example by Harrison in 1906 and Carrel et al. in 1911^{25–28}. The big breakthrough, though, happened in the 1950s with the establishment of immortalised cell lines. The isolation and cultivation of cancer cells from patient Henrietta Lacks in 1951 revolutionised the field. While ethical concerns around the lack of patient consent have been widely publicised, HeLa cells are cultured to this day and have contributed to many discoveries in the past decades^{28–30}.

Since these early days, standard cell culture protocols have been established, and refined and today build on decades of knowledge. This expertise enables researchers to reliably culture cells in 2D. Here, cells are cultured in a 2D monolayer in tissue culture dishes, often on hard plastic surfaces. The strength of standard cell culture is the relative ease of handling, with standardised reagents, equipment, as well as consumables and countless protocols, which also facilitate comparisons and collaborations between different labs.

The simplicity of this culture method is, conversely, also its major disadvantage. As discussed above, cells in tissues encounter complex and dynamic environments. While cells in monoculture can produce ECM, thus certain cell-ECM interactions are possible, 2D monolayer cell culture fails to replicate key physiological features. This leads to atypical cell behaviour and morphology, such as aberrant cell polarisation, dedifferentiation and altered drug sensitivities^{31–33}.

To overcome the limitations of 2D cell culture, various 3D model systems have been developed that accommodate specific aspects of the 3D microenvironment and the ECM. Spheroid cultures, where cells grow in direct contact with other cells, and organoid cultures that self-organise from stem cell

sources are well-established examples^{33,34}. Through microfluidics, perfusable culture systems can be designed with high precision and small working volumes. By harnessing microfluidic technologies, miniature models of specific physiological tissue aspects, so-called organ-on-a-chip systems, can be achieved³³⁻³⁵. All these techniques aim to increase the complexity of cell culture models and better replicate cell behaviour. However, some of the limitations include reduced standardisation and reproducibility³³. In addition, controlling the spatial organisation of cells and ECM components remains challenging^{33,34}. Additive manufacturing offers one approach to improve spatial control, accessibility, and reproducibility in advanced cell culture models.

Additive manufacturing in the Life Sciences

The idea of printing 3D objects was developed and presented to a wider public in the late decades of the 20th century and has since then been introduced to traditional manufacturing industries as well as users at home. The unifying idea behind 3D printing is the construction of a 3D structure in a layer-by-layer fashion based on computer-aided design (CAD). The commonly used umbrella term is additive manufacturing, and can be contrasted with classic machining technologies, which often subtract material, by for example drilling, cutting, and filing.

One of the major advantages of additive manufacturing is the facilitation of easy prototyping through quick design iterations. Additionally, intricate high-resolution structures can be produced, some of which are very hard or impossible to create using other manufacturing technologies. However, the speed, costs, and resolution vary substantially between different 3D printing technologies and applications.

In the life sciences additive manufacturing is highly valued for its versatility, ability to support small scale production (e.g., custom design of lab equipment) and the possibility to create a multitude of intricate geometries and designs. The 3D printed parts can be used for many applications including, but not limited to, visualisation (e.g., surgical planning), production of custom-made parts for experimental setups within for example cell biology (e.g., microfluidic chips), or for patient treatment (e.g., orthopaedic devices, maxillo-facial implants, and surgical guides)³⁶⁻⁴⁰.

Essentially, many 3D-printing technologies have the potential to be used for applications within the life sciences. Fused deposition modelling (FDM), also

termed fused filament fabrication (FFF), uses melt-extrusion of plastic materials to build a construct in a layer-by-layer fashion, and a commonly used material for life science applications is polylactic acid (PLA) due to its low toxicity and low cost³⁷. Stereolithography (SLA) offers higher fabrication accuracy, and the object is created through precise photopolymerisation of a liquid resin by either a laser beam or a digital light projector (DLP)³⁸. In selective laser sintering (SLS) a laser beam selectively sinters powdered materials, such as powder polymers, ceramics, or even metals, to form a 3D construct. Advantages of SLS are a high accuracy even for complex geometries and the ability to prepare larger print batches³⁹.

3D bioprinting

Definition of bioprinting

3D bioprinting originated within the field of additive manufacturing as early as 1988, with the first description of controlled positioning of cells using an inkjet printer⁴¹. Since then, it has developed into a distinct field of its own, becoming an important technology within tissue engineering and regenerative medicine, as well as a useful tool for basic research. It generally refers to the layer-by-layer deposition of biological substances, such as hydrogels, biomaterials, or cells, based on the spatial design defined in a CAD file^{2,42-44}. It is important to note that there is ongoing discussion in the field about whether the presence of living cells in the printed material should be considered a defining criterion of bioprinting, and some authors apply this more stringent definition⁴⁵. However, ASTM Standard F3659-24 defines bioprinting as “three-dimensional printing of materials (bioinks) to fabricate structured constructs for use in biological or medical applications”, where bioinks may be cell-laden, or cells may alternatively be seeded onto scaffolds after printing⁴⁶. There is sufficient consensus to delineate printing of inert materials, such as rigid scaffolds, for tissue engineering from bioprinting, which in all definition requires at least some degree of biological functionality of the bioinks. As ASTM F3659-24 is one of the few formal standards currently available in the bioprinting field, this definition will be used throughout the rest of this thesis.

Bioprinting technologies

Most bioprinting technologies originate from established 3D printing techniques which were subsequently adapted to the specific requirements of biological applications. Here I provide a short overview of the most important technologies.

One of the most extensively used bioprinting techniques is extrusion-based bioprinting (EBB). This technique was first introduced in 2002 by Landers et al.⁴⁷. In EBB, a viscous hydrogel is extruded through a nozzle as a continuous filament. Extrusion can be facilitated by a stepper-motor-driven piston, plunger, or screw, as well as pneumatic systems with or without a plunger, or solenoid systems. Its advantages are cost-effectiveness, wide choice of commercially available systems and an extensive selection of suitable bioinks^{42-44,48}. Disadvantages can be limited resolution compared to other technologies and the shear stress exerted on cells in the nozzle that may impact cell viability. Furthermore, the bioink choices are limited by the overall gelation requirements of the inks and it is beneficial to use shear-thinning hydrogels that flow viscously under shear stress, but self-heal after extrusion to regain their hydrogel structural properties^{42-44,48,49}.

Based on traditional inkjet printing, droplet-based bioprinting (DBB) generates precise droplets of bioinks and discharges them on the desired substrate. Some of the main advantages are the precise control over the dispensed volumes and spatial design, as well as the ability to print without contacting the substrate or printed construct, both of which are beneficial when building complex heterogeneous constructs composed of multiple different cell types or hydrogels. Furthermore, arrays of several printheads increase printing speeds compared to other technologies. However, known issues are clogging of the nozzles and limited bioink choices due to the constraints of the nozzle size, viscosity, and printing parameters^{44,50,51}.

Laser transfer bioprinting, specifically laser-induced forward transfer (LIFT), enables very precise nozzle-free, contactless micropatterning. Here, a laser beam selectively evaporates bioink on an energy absorbing donor substrate which leads to a droplet discharge to a receiver substrate, e.g., a hydrogel-covered glass slide. This permits single-cell resolution and tight spatial control. On the downside, the technology requires experienced users as parameter optimisation must be performed for every laser-bioink combination. Additionally, it is difficult to print larger constructs^{44,52}.

Selective photopolymerisation and light-based vat polymerisation bioprinting represent a group of technologies that use photoactivatable bioresins. This technology can be further divided into stereolithography (SLA), digital light processing (DLP), multiphoton lithography, and the relatively recently developed volumetric bioprinting. Generally speaking, selective focusing of lasers or projected light leads to spatially-defined polymerisation of the bioink in a

reservoir. This enables high-resolution printing of complex geometries. The key differences between these technologies lie in how structures are built, specifically point-by-point, layer-by-layer, or volumetric, as well as the light source. Disadvantages include a limited selection of photocurable bioresins and the associated cytocompatibility issues of photoinitiators; light scattering through high cell density or in the presence of ECM molecules; and increased complexity for multimaterial printing. However, the recent developments in volumetric bioprinting have greatly enhanced previous limitations such as speed and throughput^{44,53}.

Bioinks and hydrogels

Bioink is the term used to describe the biological material deposited during bioprinting. As with bioprinting in general, there is currently no universal consensus regarding the exact definition of bioink. Some authors argue that only inks containing living cells should be classified as bioinks, whereas cell-free inks should be called biomaterial inks^{54,55}. The ASTM Standard F3659-24, however, highlights the bioactivity of bioinks as a defining factor rather than the presence of encapsulated cells, as cells may also be added after the printing process is completed⁴⁶. In Study II and IV of this thesis, the term bioink refers to extrudable hydrogel-based biomaterials of biological origin, including collagen and laminin-containing inks.

In recent years, the development of bioinks has progressed substantially, and increasing commercial availability has expanded the range of available bioinks. The choice of bioink depends considerably on the specific constraints of the chosen bioprinting technologies and desired target tissue. Mechanical integrity, rheological properties governing printability and chemical properties such as crosslinking ability and, of course, biological compatibility need to fit the demands of the project at hand^{55,56}.

Most bioinks are based on hydrogels, which are essentially water-swollen networks of polymers. Broadly, bioinks can be divided into naturally derived, synthetic or hybrid hydrogels. These materials can have various crosslinking and polymerisation modalities, including thermal, ionic, enzymatic, or photo-induced crosslinking, resulting in covalent or noncovalent crosslinking of the gels. Additional chemical modification with, for instance, methacrylate groups, which initiate polymerisation after stimulation of photoinitiators, can enhance the repertoire of hydrogels³¹. Common hydrogels used in bioprinting are collagen, fibrinogen, alginate, hyaluronic acid, silk, gelatine and other natural polymers⁵⁶.

As discussed previously, collagen is one of the primary structural components of the ECM in mammalian tissues and is thus a particularly appealing choice for modelling tissue microenvironments due to its great biocompatibility^{18,57,58}. Both collagen and its denatured form gelatine offer amino acid motifs for cell attachment, typically via integrins, and thereby facilitating cell-matrix interactions within the bioink^{18,58,59}. In addition, collagen plays an important role in processes such as bone healing and ECM remodelling during disease progression, including cancer, further supporting its relevance for bioprinting applications^{18,20,57,58,60,61}. Two different collagen bioinks were used in Study I and II, respectively.

Collagen used for tissue engineering applications is typically sourced from bovine, porcine, rat or aquatic animal tissues, although human sources are also available. Mammalian-derived collagen retains the triple helical structure and closely resembles human collagen. However, aside from ethical considerations, mammalian collagen may induce immunological reactions when implanted. Additionally, batch-to-batch variation needs to be considered⁵⁵.

Collagen is soluble under acidic conditions but undergoes fibrillar self-assembly when the pH is neutralised, a property that is commonly harnessed for hydrogel formation^{7,57,60,62}. However, collagen hydrogels typically exhibit limited mechanical stability. Mechanical properties are strongly influenced by collagen concentration and the mode of crosslinking. To improve mechanical stability, various chemical modifications have therefore been developed. For example, methacrylated collagen (ColMA) contains photoactive methacrylate groups that enable photopolymerisation⁵⁷. Similarly, gelatine can be modified to form GelMA, which contains methacrylamide and methacrylate groups⁶³. Alternatively, increasing collagen concentration can enhance mechanical strength and is also physiologically more relevant, for example, the increased collagen concentrations in tumours⁶⁴. However, higher concentrations often reduce printability and cell compatibility⁵⁷. Embedded bioprinting approaches offer good opportunities to overcome these limitations and enable printing of collagen inks with high accuracy, as will be described in more detail below.

Norein et al. recently developed a dense collagen-silica (DCSi) formulation with high mechanical strength. This hybrid bioink contains 16% collagen, which retains the fibrillar collagen architecture, and the silica component guides crosslinking and condensation. The resulting constructs show high structural stability and fidelity, which lead to lower degradation of printed DCSi scaffolds *in vivo*

than commercial collagen constructs. This highlights the suitability of this ink for hard tissue applications. While the printing process is not compatible with cell encapsulation, due to large pH changes, the fibrillar collagen supports robust cell adhesion⁶⁵. This ink was used in study IV.

Depending on experimental design, other hydrogels and bioink compositions can be advantageous. Laminin-containing inks, for example, can be used to tune the ECM composition for tissue-specificity and to influence cell differentiation or stemness^{66,67}. Apart from hydrogels, bioinks can also contain various biomaterials, such as hydroxyapatite or other calcium phosphate derivatives, signalling molecules and different bioactive materials. Furthermore, high-density cell suspensions that are deposited as either pellets, cell aggregates or spheroids are also considered bioinks without being suspended in hydrogels⁵⁶.

In conclusion, the choice of bioink and whether cells are encapsulated or seeded after the print is determined by the specific material properties, the desired biological model, the printing technology and the crosslinking strategies.

Challenges in bioprinting and current technological advances

3D bioprinting enables the creation of complex 3D hydrogel models. The technological development is progressing rapidly, with ongoing refinement of existing technologies. However, bioprinting is often associated with the goal of printing entire organs as “spare parts” for patients. While this may be feasible at some point in the distant future, the reproduction of the size and complexity of entire human organs is simply not achievable with current technologies. Therefore, exaggerated claims regarding the imminent printing of fully functional organs should be interpreted with caution. However, 3D bioprinting of biomimetic tissue constructs, which do not aim to incorporate the entire complexity of organs but rather focus on mimicking certain aspects of tissues, has advanced considerably in recent years^{36,68,69}. Major hurdles in bioprinting living cellular constructs for medical use include, among others: control of mechanical properties, emulating physiological heterogeneity, current limitations in the expansion of functional cells from stem cells, maturation of the bioprinted constructs, interfacing bioprinted tissues with the physiological vasculature and regulatory aspects for clinical applications^{40,44,68,69}.

While first clinical applications of 3D bioprinted implants are being explored in upcoming clinical trials^{44,69}, the current strength of 3D bioprinting lies, without doubt, in its use to model three-dimensional tissue landscapes for *in vitro* studies as well as for testing new approaches in tissue

engineering. Overall, 3D bioprinting has been widely adopted by the research community in the past decade, which is reflected in the increasing number of annual publications in the field. These increased from 261 publications in 2016 to 1 522 publications in 2025 based on a PubMed search using the keyword “bioprinting”⁷⁰.

Many of these publications aim to address the technological limitations, and thus a comprehensive discussion of all ongoing technological advances is beyond the scope of this thesis. Therefore, only selected examples will be discussed to illustrate how current limitations in bioprinting are being addressed.

Embedded bioprinting addresses some of the major limitations of extrusion bioprinting, specifically the low resolution of printed constructs and the need for support materials. One example of embedded bioprinting is freeform reversible embedding of suspended hydrogels (FRESH). FRESH is a technique where the bioink is extruded into a yield stress support bath which may also facilitate bioink crosslinking depending on the chosen materials^{71,72}. In 2019, Lee et al. demonstrated that their refined FRESH approach increased the resolution of extruded hydrogels to up to 20 μm . They used acidified collagen type I as a bioink and extruded it into a gelatine microparticle support bath. Due to the pH buffer in the support bath the acidified collagen encounters a rapid pH change which leads to gelation of the collagen hydrogel. The gelatine support bath provides structural support throughout the print but can easily be melted away at 37 °C once the printed construct is fully solidified. Prints of different sizes were demonstrated including fine channels to mimic blood capillaries and a full-size model of a human neonatal heart. Additionally, they demonstrated the ability to incorporate high-concentration cell-containing inks within a FRESH print with cells retaining high cell viability⁷³. The FRESH printing technology is easily adapted to other bioinks, such as alginate, fibrinogen or methacrylated gelatine through changing the components of the support bath to the relevant gelation mechanism⁷². Recent studies showed FRESH applications for printing other ECM molecules such as collagen type II and III or to create perfusable ECM containing scaffolds, termed CHIPS^{74,75}

The topic of high cellular density and perfusability needed for tissue engineering approaches was addressed in a study by Skylar-Scott et al. Here they started with producing large quantities of so-called organ building blocks (OBBs) from organoid cultures, which are subsequently compacted to form a dense living suspension. This version of embedded bioprinting introduces sacrificial ink into this dense matrix. After removal of this sacrificial inks,

perfusible channels remain. This technique, termed sacrificial writing into functional tissues (SWIFT), leads to a densely cellularised patient- and organ specific tissue, which matures when cultured under perfusion ⁷⁶.

The replication of heterogeneous native tissues is a challenge which will require use of several different approaches. Multimaterial printing is a way forward to increase complexity and can be achieved with many different bioprinting solutions. High speed printing of several materials was shown by Skylar-Scott et al. through using multimaterial multinozzle 3D (MM3D) printing, which generated individual voxels, the 3D analogue of a pixel, and thus quickly produced 3D objects with spatially programmed structure, composition and properties ⁷⁷. Another way to model heterogeneity is the use of bioprinted gradients. For instance, Kuzucu et al. used extrusion bioprinting to form gradients of stiffness, cell density and immobilised peptides in their carboxylated agarose bioink ⁷⁸. Another way of creating composite structures is continuous chaotic bioprinting which results in reproducible lamellar patterns ⁷⁹.

Another noteworthy recent advance is the development of volumetric bioprinting in which objects are built by tomographic reconstruction within seconds, rather than a conventional layer-by-layer approach ^{44,53}. Related vat-based light polymerisation technologies, such as Filamented Light (FLight) further expand the capabilities of this class of technologies and show great potential for many applications within tissue engineering ⁸⁰. Additionally, 4D bioprinting is an approach in which 3D bioprinted scaffolds are induced to change shape in response to internal or external stimuli, enabling the tissue constructs to change over time ⁸¹.

Overall, the rapid development of bioprinting is continuously generating new technological advances that are likely to further expand the capabilities of the field and overcome current limitations especially in applications such as *in vitro* drug testing and bone tissue engineering.

Anti-cancer drug combination testing in 2D and 3D

Cancer is a broad group of diseases characterised by uncontrolled cell proliferation and the ability of abnormal cells to invade surrounding tissues and spread to distant organs. Breast cancer remains the most prevalent malignancy among women worldwide, with an estimated 2.3 million new cases and approximately 660,000 associated deaths reported in 2022 ⁸². If discovered at an early stage, as a non-metastatic tumour, breast cancer has a relatively good

prognosis and is curable in 70-80% of cases⁸³. However, triple-negative breast cancer which cannot be treated with hormone-based or HER2-targeted therapies has markedly poorer treatment outcomes, indicating a need for better treatment strategies^{84,85}.

Cancer treatment is largely based on three pillars, namely surgery to remove the primary tumour, radiotherapy, and anti-cancer drug therapy, with the goal of selectively killing the neoplastic cells with as limited side effects as possible. In breast cancer, the treatment choice depends greatly on the subtype and stage, the overall health of the patient and whether the patient is menopausal. Therefore, there is no universally applicable treatment plan, but treatment options are evaluated on a patient-specific basis^{83,86}.

Combination therapies

Common treatment plans for breast cancer involve combinatorial drug therapies⁸⁶. By treating patients with combinatorial drug therapies and targeting different signalling pathways, the effectiveness of the overall therapy can be increased, which in turn can lead to lower dosages. This reduces toxic side effects, which are well-known problems in chemotherapy and significantly impact patients' quality of life⁸⁶.

An additional challenge in cancer therapy is the prevalent heterogeneity in cancer. Intratumour heterogeneity, which describes the variability of cell responses within tumours, can promote drug resistance through the selective survival of resistant cell populations^{87,88}. Conversely, intertumour heterogeneity refers to the biological variability in different patients, which complicates the ability to reliably predict therapeutic response⁸⁷.

Therefore, some effects of combinatorial drug treatments can be attributed to independent drug action, alternatively they can be signs of synergy, additivity or antagonism^{87,89}. Synergy occurs when the combined effect of therapies exceeds the sum of their individual effects, whereas additivity is observed when the combined effect equals the sum of their individual effects. In contrast, mild antagonism describes a combined effect that is lower than the expected sum but may still be greater than the effects of each treatment alone^{90,91}. One way of mathematically predicting the combined effect of two drugs is the Bliss Independence model, which was used in Study I^{90,91}.

Overall, identifying beneficial drug combinations could provide substantial benefits for patients, but remains challenging due to tumour heterogeneity, highlighting the need for improved combinatorial drug testing.

Combinatorial drug testing

Common methods for screening drug combinations are often 2D plate-based assays that test drug combinations in a dose-response matrix. Advantages of plate-based approaches include their ability to scale for high throughput and automation. However, screening many drug combinations and concentrations is still associated with high cost and low success in identifying synergistic interactions⁹². For instance, a large-scale study in 2015 used over 1000 patient derived xenograft (PDX) models to predict clinical treatment outcomes for 38 compounds. They found that combination therapies were generally more successful than single-compound treatment⁹³. However, a follow up study found that only a few of these combinations were truly synergistic. Other beneficial combinations could be explained by independent drug action⁸⁷.

A recent large-scale screening of 2025 drug combinations on 125 cell lines of breast, colorectal and pancreatic cancer yielded a total of 108 259 drug combination-cell line pairs. Among these pairs, synergy was observed in 5.2% of all tested cancer types, with 4.4% of the synergistic combinations occurring in breast cancer⁹⁴. This corresponds with findings that most of the FDA-approved drug combination therapies are based on additivity⁹⁵.

These examples show that synergy, additivity, and independent drug action are important yet complex topics in cancer therapies. Clearly, better strategies to identify suitable drug combinations are required. While high-throughput 2D approaches are appropriate to identify suitable candidates, they lack many physiologically relevant features. Moreover, due to ethical considerations, large-scale screening in animal models also needs to be limited. In this context, advanced 3D cell culture models can bridge the gap and provide valuable insight.

Bone tissue

Current clinical needs in fracture repair

Bone fractures are among the most common traumatic injuries. According to the Global Burden of Disease study, 178 million fractures occurred in 2019, highlighting the overall prevalence of fractures⁹⁶.

Fracture treatment primarily involves mechanical fixation either externally with a cast or internally through metallic hardware, such as screws or plates. The goal is to align the broken bones and stabilise the fracture site sufficiently for healing. However, unsatisfactory treatment outcomes, caused by molecular disruption of the healing process or by large bone defects, so-called critical size defects, result in so called “non-unions” and demonstrate a persistent need for improved fracture management ^{97,98}. Understanding the biological processes governing bone regeneration is therefore essential for developing improved therapeutic strategies.

General physiology of bone

Bone tissue may be mistaken for a relatively inert tissue due to its rigidity and high mechanical strength; however, this mineralised connective tissue is highly dynamic and undergoes constant remodelling throughout life. Bone can be categorised as either dense, solid cortical bone or trabecular bone, which is characterised by its honeycomb-like structure ^{99,100}. It consists of organic and inorganic matrix components, as well as several types of bone cells, such as osteoblasts, osteocytes, osteoclasts, and bone-lining cells. These cells regulate bone matrix secretion, mineralisation, and bone remodelling in response to biochemical and mechanical cues ^{99–101}.

The inorganic component of bone consists mainly of calcium and phosphate in the form of hydroxyapatite. It provides stability and strength to the tissue. Additionally, it serves as a major reservoir of ions, specifically calcium and phosphorus, and maintains the concentrations of these ions in the body ^{99–102}.

The organic matrix is made up largely of collagen, which is often reported to account for approximately 90% of the organic matrix ^{99–102}. A recent study, however, measured that collagen corresponds to only 25–35% of total bone protein levels in mice, with collagen type I being the most abundant ¹⁷. Other components of the organic matrix include non-collagenous proteins, such as serum-derived proteins, proteoglycans, glycoproteins, glycoproteins containing an RGD sequence, γ -carboxylated (Gla) proteins and proteins of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family ^{100,102}.

New organic matrix proteins are deposited by osteoblasts. These cells develop from mesenchymal stem cells via osteoprogenitor cells to preosteoblasts and finally osteoblasts. Preosteoblasts are defined by the expression of Runt-related transcription factor 2 (RUNX2), collagen type I (Col1A1), and alkaline phosphatase (ALP). Mature osteoblasts are defined by increased expression

of osterix and RUNX2^{101,103,104}. The newly formed bone matrix is subsequently mineralised by the formation of hydroxyapatite crystals^{100,101}. This entraps the mature osteoblasts which eventually either undergo apoptosis or further differentiate into osteocytes or bone-lining cells^{103,105}.

Osteocytes, the most abundant bone cells, are embedded in the mineralised matrix and have multiple long cellular processes that form cell–cell connections in an interconnected network of canaliculi^{100,103,105}. This enables their main functions, including mechanosensation and interactions with osteoblasts and osteoclasts to regulate bone turnover. Additionally, they can directly hydrolyse the bone matrix. Thus, they are key players in bone formation and resorption^{99,101,105–107}. Osteoclasts are multinucleated cells that originate from the haematopoietic monocyte-macrophage lineage and are responsible for bone resorption^{100,108}.

During bone remodelling, temporary anatomical structures, so-called basic multicellular units (BMUs), consisting of several cell types, including osteoclasts and osteoblasts, are formed^{101,108}. In a coordinated interplay between all involved cells, old bone is resorbed by osteoclasts and new bone is deposited by osteoblasts. Remodelling occurs throughout life and is important to maintain the strength and functionality of bone tissue over time^{100,108}.

Bone healing

Bone has an impressive regenerative potential that enables true tissue regeneration after injury, restoring it to its pre-injury state. Bone healing is an intricate process that can be broadly divided into the following, overlapping stages: coagulation and pro-inflammatory phase, anti-inflammatory and angiogenesis phase, soft callus phase, hard callus phase and finally remodelling phase^{97,109}. In essence, an inflammatory response is triggered, new tissue is deposited, leading to an initial increase in tissue volume, and finally, the newly formed bone is remodelled⁹⁷. While these processes normally lead to successful bone regeneration, approximately 5–10% of fractures result in non-union and require additional fracture management^{97,110}.

Options to enhance healing may include biophysical stimulation, local treatments with osteoconductive or osteoinductive materials or systemic stimulation⁹⁷. Osteoconductive materials support the growth of bone along and into the material without affecting cell differentiation, while osteoinductive materials promote cell differentiation towards the osteoblastic lineage¹¹¹. Biomaterials for guiding bone healing are an active field of research and many

materials are already used clinically. However, resorbable osteoconductive materials that also support fracture fixation, such as bone adhesives, are still needed in the clinic ¹¹².

Aims

The overall aim of the studies presented in this thesis was to explore how additive manufacturing could be used to facilitate the transition from 2D to 3D cell culture models. The studies focused on two main topics. Firstly, the development of methods to generate novel cell culture models (Studies I and II). Secondly, the application of different cell culture models that bridge 2D and 3D systems to study cellular responses to drugs or biomaterials (Studies I, III and IV).

The specific aims of the studies were:

- Study I To introduce a cell culture device for *in vitro* combinatorial anti-cancer drug testing and to establish a protocol and imaging strategy to evaluate cell responses to overlapping, dynamic drug gradients generated by diffusion of drugs from drug-containing cell culture inserts.

- Study II To establish an open source bioprinter based on the E3D motion system and tool changer and to provide proof-of-concept of multimaterial printing, bioprinting of cell-laden bioinks and high-resolution FRESH bioprinting.

- Study III To investigate the biosafety of a novel bone adhesive based on phosphoserine modified calcium phosphate cements *in vitro* and *in vivo* as well as to assess gene expression responses.

- Study IV To explore the osteogenic potential of 3D bioprinted phosphoserine-soaked mineralised collagen scaffolds *in vivo* and to evaluate the cell response to these materials *in vitro*.

Methods

Cell culture

Breast cancer cell lines

The triple-negative breast cancer cell line, MDA-MB-231, was established in the 1970s from a 51-year-old patient and has since become one of the most commonly used cell lines for *in vitro* investigations of triple-negative breast cancer¹¹³. MDA-MB-231 cells are mesenchymal-like breast adenocarcinoma cells, triple-negative (lacking expression of receptors for estrogen, progesterone, and human epidermal growth factor 2), migratory and invasive cells. They are commonly used to study novel drug therapies for triple-negative breast cancer. They can also be grown in 3D and used for xenograft models^{114–118}. MDA-MB-231 cells are easy to grow in standard 2D conditions (37 °C, 5% CO₂) in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX, supplemented with 10% fetal bovine serum (FBS) and, if necessary, 1% penicillin–streptomycin (PenStrep). MDA-MB-231 cells were used in Studies I and II.

Bone cell lines

There is a wide choice of different bone cell models available, both primary cells as well as established cell lines. We chose to use a preosteoblast cell line for most cell experiments in Study III and Study IV. Preosteoblasts are cells of the bone lineage that are not yet fully differentiated to osteoblasts. Therefore, they retain differentiation potential but can secrete ECM molecules, such as collagen, and to some extent mineralise the bone matrix. We selected the widely used mouse preosteoblast cell line, MC3T3-E1, which is derived from new born calvarial bone and was first described in 1981¹¹⁹. MC3T3-E1 cells express high levels of alkaline phosphatase (ALP) in confluent conditions and maintain the ability to excrete and mineralise matrix when cultured with induction media, for example containing ascorbic acid and inorganic phosphate^{120–122}. However, it is important to note that differentiation and mineralisation potential vary between the different available subclones. Thus, to ensure comparability between experimental results, it is of utmost importance to also

report which subclone was used ^{121,123}. For our studies, we selected subclone 4, which maintains a high mineralisation potential ^{121,123}. MC3T3-E1 cells are easy to culture under standard conditions (37 °C, 5% CO₂) in alpha MEM supplemented with 10% FBS and, if necessary, 1% PenStrep. However, it has been shown that continuous passaging negatively influences the osteogenic potential of MC3T3-E1 cells after passage 30 ¹²⁴. Although we did not perform differentiation studies, all our experiments were performed on MC3T3-E1 subclone 4 cells at passages below 30.

In Study IV, rat mesenchymal stem cells (rMSCs) were also used to assess cell–material interactions. As osteoblasts differentiate from MSCs, they represent a less committed cell population within the osteogenic lineage ^{101,103,125}. *In vivo*, MSCs reside in the bone marrow and contribute to bone regeneration, making them relevant for studying interactions with implanted biomaterials. Their response may therefore better reflect *in vivo* behaviour compared to more differentiated cells. MSCs are a multipotent cell population capable of differentiating into osteogenic, chondrogenic, and adipogenic lineages ^{125,126}.

Measuring cell viability

Cell viability assays are used to assess cellular responses to drugs or material exposure, providing information about cytotoxicity, proliferation, and general health of a cell population. Common indicators of cell health or lack thereof include e.g., membrane integrity, enzymatic or metabolic activity. These parameters can be measured in different ways, for instance through brightfield or fluorescence microscopy, flow cytometry, or plate-based assays that utilise colorimetric, fluorescent, or luminescent reagents ¹²⁷. The choice of viability assay depends on several experimental considerations, including the number of samples (i.e., is high throughput necessary), whether the test is performed at an experimental endpoint or requires continuous monitoring, and whether the cells are cultured in 2D or 3D cell culture systems. Furthermore, the presence of (bio)materials or drugs may impact the assay readout and this needs to be tested and controlled for in each experimental setup.

Plate-based assays have the advantage of protocols amenable to high-throughput and relatively easy data analysis. However, the readouts are often indirect indicators of cell health on a population level, such as metabolic parameters. Since these assays do not directly quantify cell number, metabolic readouts may be misinterpreted if a small number of cells have particularly high metabolic activity.

Cell viability assessment via microscopy can give single-cell resolution and provide additional information such as cell distribution or morphology. On the other hand, these approaches can be more labour-intensive during both image acquisition as well as image analysis.

2D cell viability assay - PrestoBlue assay

Metabolic activity can be measured by assessing the cellular redox capacity. PrestoBlue (Invitrogen, Thermo Fisher), used in Study I, II and IV, is one of several commercially available resazurin-based cell viability assays. The initially blue compound resazurin is cell-permeable and non-toxic. In viable, metabolically active cells, resazurin is enzymatically reduced to resorufin, which is a pink compound with a strong fluorescent signal¹²⁷. This conversion can be measured by absorbance or fluorescence on a microplate reader. The PrestoBlue protocol is simple and does not require cell lysis, allowing repeated measurements on the same sample. According to the manufacturer's instructions, it should give results in as little as 10 minutes. In our studies, however, longer incubation times (approximately 3 hours) produced more consistent signals for the specific cell types and seeding densities used. It has been reported that PrestoBlue may yield inaccurate measurements in larger 3D constructs and certain hydrogel formulations¹²⁸. Therefore, the PrestoBlue assay was used exclusively to assess viability in 2D cultures.

It is also important to keep in mind that while a decrease in metabolic activity is considered an indicator of reduced cell health, other factors such as drugs, biomaterials or other components in the cell media, may influence the reduction of resazurin. Therefore, the inclusion of appropriate experimental controls is crucial^{127,129}.

3D cell viability assay - CellTiter-Glo 3D viability assay

In 3D cell constructs, the measurement of cell viability may be complicated by the presence of hydrogels that may bind assay reagents, or the size of constructs that may limit the perfusion of reagents, leading to potentially inaccurate results^{127,128,130}. The CellTiter-Glo 3D viability assay (Promega) was designed for improved sensitivity in 3D cultures and delivered more reliable cell viability data when compared to other cell viability tests, specifically on collagen scaffolds^{128,131}.

The CellTiter-Glo 3D assay is also a metabolic assay, measuring the ATP content. ATP levels as a measurement of cell viability have been shown to

correlate with the cell number of a sample. After cell death ATP is rapidly depleted and thus serves as an indicator of metabolically active cells ¹³¹⁻¹³³.

The assay protocol requires only one assay solution, which lyses the cells after which a luminescence signal is generated by luciferase in the presence of ATP ¹³³. This luminescence signal is collected using a microplate reader. However, as this is an endpoint assay, the samples cannot be reused and thus continuous monitoring of a culture is not possible.

In study IV, we used the CellTiter-Glo 3D assay to assess cell viability on bioprinted collagen scaffolds.

Microscopy

In confocal fluorescence microscopy, laser light is used to illuminate the sample, and a pinhole aperture only permits in-focus light to pass to the detector. This enables high-resolution imaging and optical sectioning of 3D samples. In laser scanning microscopes (LSM), the image is built by scanning the sample point-by-point in an x-y direction. By changing the focal point in the z direction, optical sections, so-called z-stacks, can be obtained and assembled into a 3D image ^{134,135}. This means that a confocal microscope can be used for imaging a wide range of samples, including hydrogel scaffolds. However, the presence of hydrogels can limit the depth of laser penetration through light scattering and absorption. Additionally, there may be overlap between the fluorescence signals for specific cell stains and the material. Therefore, the choice of fluorescent reporters needs to be evaluated in all experiments including biomaterials and hydrogels. For example, this limited the use of the nuclear marker (NucBlue) in several experiments.

All confocal microscopy was performed using an LSM 700 confocal microscope (Zeiss, Jena, Germany) with the specific objective and imaging settings reported in the respective studies. Image analysis was performed using the FIJI version of ImageJ ¹³⁶, as described in the corresponding studies.

Live/Dead imaging

Through live/dead staining, cell viability is directly assessed at single-cell resolution using microscopy, compared to microplate reader-based assays that average signal from the entire cell population in each well. In Study I and II, we used fluorescein diacetate (FDA) and in Study IV, calcein acetoxymethyl

ester (Calcein-AM) to identify living cells and propidium iodide (PI) to identify dead cells. PI permeates dead cells with disrupted membrane integrity and intercalates into DNA. This leads to an increase in PI fluorescence, which has excitation/emission maximum at 535/617 nm. FDA is actively hydrolysed to fluorescein (excitation/ emission maximum 490/520 nm) in living cells and is retained in cells with intact membranes. However, FDA will be removed from cells over time, resulting in loss of signal during longer experiments^{127,137}. Calcein-AM is similarly actively hydrolysed intracellularly to the fluorescent molecule calcein (excitation/emission maximum 495/515 nm) but is retained longer in viable cells¹²⁷. Both live/dead protocols are live imaging assays which can be performed on cells grown in 2D as well as encapsulated cells. When cells were suspended within a matrix, we adjusted the concentrations and incubation periods of the cell staining as necessary. Nuclear staining, using NucBlue (Invitrogen, Thermo Fisher), was also added in certain experiments to identify all cells in the population.

Apoptosis assay

To assess modes of cell death, we employed a double staining strategy using an apoptosis marker (Caspase 3/7) and a cell death marker (PI). This was performed in Study I and Study II. During apoptosis caspase 3 and 7 are active and can cleave the peptide sequence of the otherwise nonfluorescent CellEvent Caspase 3/7 reporter (Invitrogen, Thermo Fisher), resulting in a fluorescent signal (excitation/emission maximum 502/530 nm) if the cells are undergoing apoptosis¹²⁷. This was combined with PI staining as described above. The double staining enables cell death detection independent of the mode of cell death, which may be especially beneficial when working with drugs for which the mode of action is unclear. Depending on the specific experimental setup we also used a nuclear marker, NucBlue (Invitrogen, Thermo Fisher), to detect all cells in a sample. As with the live/dead staining protocols, adjustments of the concentrations and incubation periods were made as necessary.

Additive manufacturing

Design and prototyping of CombiCTx inserts

The CombiCTx assay was inspired by the patented CombiANT assay¹³⁸ for antibiotic interaction testing. In this assay, the CombiANT insert, filled with antibiotic drugs, is placed at the bottom of agar plates, which are subsequently streaked with a bacterial suspension. The assay readout is based on the

identification of the edges of bacterial growth zones. However, in the CombiCTx assay we aimed to investigate the effects of anti-cancer drug combinations on human cells and thus the assay needed to be adapted to the constraints of adherent cell culture. This also affected the design of the cell culture insert. For instance, the assay was flipped upside down compared to the CombiANT setup. This required the cell culture insert to have support struts to maintain a consistent distance from the adherent cells on the bottom of the well. The final CombiCTx insert fits into standard 6-well tissue culture plates, and the drug reservoir volume is 124 μL . Consequently, the insert dimensions were modified compared with the original design.

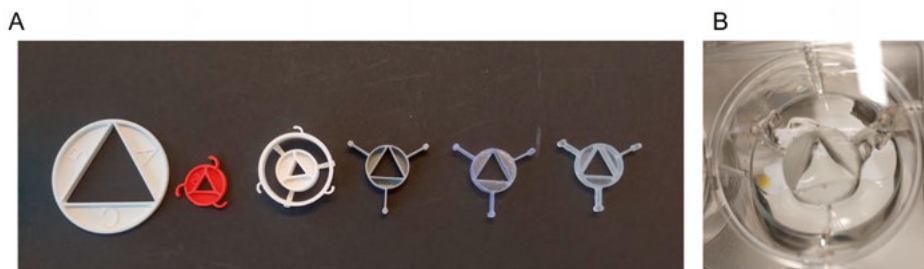


Figure 1. CombiCTx design process. **(A)** Design evolution from the CombiANT design (far left) to the final CombiCTx device (far right) and various design iterations in between. **(B)** Example of a failed CombiCTx attempt where the gel layer ripped due to swelling of the 3D printed cell culture insert

The computer-aided design (CAD) software Autodesk Fusion 360 (Autodesk Inc.) was used to design the CombiCTx insert. Prototyping was done using different 3D printing technologies and materials, as seen in Figure 1A. Initially, fast and easy design iterations were desired, thus FDM printing using X-PLA filaments was chosen. The downside of this technique was printing artefacts, such as improperly fused filaments caused presumably during the printing process, that led to drug leakage during assay setup. Furthermore, PLA can be sterilised with 70% ethanol and UV irradiation, but it cannot be autoclaved. Therefore, other (autoclavable) materials and printing technologies with higher resolution, were tested. However, several tested resins and materials were autofluorescent or otherwise impaired imaging of the cells. Additionally, during assay development, warping of the gel layer, which led to disturbances of the gradients, was repeatedly observed (Figure 1B). Investigation of the issue identified swelling of the 3D-printed inserts as the cause. Thus, once the design was finalised, the CombiCTx inserts were produced through CNC milling from 8 mm thick polycarbonate sheets (Figure 1A, right-most insert). These inserts are easy to sterilise by autoclaving, the transparent material does not swell and is compatible with fluorescence microscopy.

Extrusion based bioprinting

In Study II, all bioprinting was performed using the open source bioprinter described in that study. The specific constructs were designed with the CAD software Autodesk Fusion 360 (Autodesk Inc.) which enables precise drawings of the construct's geometries. Different geometries were chosen depending on the experimental setup, e.g., solid squares to facilitate cell seeding or multimaterial printing etc. Once the design was completed, a .stl-file was exported. To set print parameters and generate the toolpath Simplified 3D software (Simplified3D) was used. This process is called slicing and generates the G-code. Finally, the G-code files were uploaded to the user interface powered by the Duet Wifi Controller. The specific print parameters, i.e., printing speed, layer height, nozzle size, etc., were chosen based on the bioink, the desired geometry, and whether cells were encapsulated in the ink.

For FRESH bioprinting, the collagen bioink LifeInk 240 (Advanced Biomatrix) was extruded into a gelatine support bath (LifeSupport, Cellink). For easier handling, the scaffolds were printed into small custom designed baskets. After printing, the support bath was melted away at 37 °C in the incubator and washed in PBS prior to cell seeding.

The laminin containing ink (Laminink+, Cellink) supported cell encapsulation. Therefore, MDA-MB-231 cells were mixed into the ink as per the manufacturer's instructions. The cell laden ink was printed with a 18G nozzle and scaffolds were submerged in crosslinking agent (Cellink) for 1 minute. Thereafter, the scaffolds were cultured under standard conditions.

In study IV, all bioprinting was performed using BIOX (Cellink), a pneumatic extrusion bioprinter. The dense collagen bioink, DCSi, was prepared as described in the manuscript and in more detail by Norein et al.⁶⁵. Using a 20G nozzle and a cooled printhead as well as print bed (10 °C), single-layer constructs were printed as 10 x10 mm sheets for *in vivo* experiments or 20 x 20 mm sheets for *in vitro* experiments. Immediately after the print, scaffolds were submerged in borate buffer (0.6 M, pH 10, 30 °C) for crosslinking. For *in vitro* experiments, discs of 6 mm diameter were prepared to fit into standard 96-well plates. Prior to cell seeding or implantation, all scaffolds were washed in PBS and remained either untreated, were mineralised (MDCSi) or soaked in phosphoserine (pSER) solution with or without prior mineralisation (MDCSi +x% pSER and DCSi +x% pSER). Figure 4 in the discussion section for Study IV gives an overview of the different scaffold treatments. For mineralisation the samples were treated according to a rapid mineralisation protocol¹³⁹ as described in the manuscript in more

details. The pSER treatment was performed directly before cell seeding. To this end, samples were soaked in pSER solution at pH 7, for 2 hours. The exact pSER concentrations are specified in the manuscript.

In vivo assessment of novel biomaterials

While 3D models can mimic certain aspects of tissues, they remain simplified models. Therefore, *in vivo* experiments are still necessary to assess the systemic response to biomaterials in a physiological environment before human applications can be considered. *In vivo* models provide important features such as the complex interplay between different cell types and signalling pathways, a fully competent immune system, and physiological processes including vascularisation and fluid exchange. Thus, selected *in vivo* experiments were performed to evaluate the biological response to the investigated biomaterials. All animal experiments were performed in accordance with relevant guidelines and under the following ethical permits by the Uppsala Committee of Animal Research Ethics, approval number 5.8.18-11832/2017 and 5.8.18-20773/2021.

In Study III, a subcutaneous model in rats was used to assess the safety of a novel bone adhesive and to identify any adverse immune reactions such as implant rejection. The site of implantation was chosen to detect any ectopic bone induction, which is not desirable in this class of osteoconductive biomaterials intended for gluing bone fragments. The implants were placed in subcutaneous pouches on the rat's back, with six implants per animal allowing direct comparison between formulation groups. Animals were sacrificed 6 and 12 weeks post-implantation.

In Study IV, bone healing in response to the modified DCSi scaffolds was evaluated using a unicortical defect model in rats. In this model, a non-critical size defect is drilled in each femur, enabling the paired observation of two treatments within the same animal. Additionally, no external stabilisation is required¹⁴⁰. This design allows direct comparison between treatment groups while reducing the number of animals required, in accordance with the principles of the 3Rs. The rats were euthanised after 6 weeks. For high resolution 3D imaging of the femurs, micro-computed tomography (μ CT) was used to quantify new bone formation and defect healing.

To further characterise tissue responses to the implanted materials, histological analysis was performed in both studies. Haematoxylin and eosin staining was

performed in Study III and IV, resulting in purple staining of nuclei and pink staining of cytoplasm and ECM. In Study III, additional samples were assessed using hard histology where decalcification of the tissue is not necessary.

Gene expression analysis

Gene expression from tissue samples can be analysed using qPCR. In Study III, this was used to assess the expression of genes associated with inflammatory responses and osteogenic differentiation after subcutaneous implantation of a bone adhesive in rats. Thus, we aimed to investigate any undesired ectopic effects of this material in soft tissue. The tissue samples were collected at 6 and 12 weeks post-implantation.

To investigate potential ectopic osteogenic activity and inflammatory responses, the expression of six genes associated with osteogenic differentiation and inflammation was analysed using commercially available TaqMan assays (Applied Biosystems).

The osteogenic markers included collagen type I alpha 1 chain (Col1a1, TaqMan assay ID Rn01463848_m1), SRY box 9 (Sox9, TaqMan assay ID Rn01751070_m) and Runt-related transcription factor 2 (Runx2, TaqMan assay ID Rn01512298_m1). These genes are well-established osteogenic markers that play key roles at different stages of the osteoblast lineage, with Runx2 in particular being implicated in ectopic bone formation^{103,141–143}.

Inflammatory responses were evaluated by analysing tumour necrosis factor (Tnf, TaqMan assay ID Rn99999017_m1), interleukin-1 beta (Il1b, TaqMan assay ID Rn00580432_m1) and interleukin-6 (Il6, TaqMan assay ID Rn01410330_m1), cytokines whose expression is implicated in increased inflammatory responses^{144–146}. Additionally, glyceraldehyde-3-phosphate dehydrogenase (Gapdh, TaqMan assay ID Rn01775763_g1) was used as a reference gene to normalise the data using the $2^{-\Delta\Delta C_T}$ method.

This experiment was performed as an add-on experiment after samples were already collected for histological evaluation. Considering the 3Rs principle, it was chosen to collect gene expression data from these samples to maximise data output from *in vivo* experiments. However, the design of this experiment could be improved by using multiple reference genes and validating their stable expression in this specific model as recommended by the MIQE guidelines^{147–149}. Alternatively, a spatially resolved gene expression analysis, such as spatial transcriptomics, could provide site- and cell-specific expression data, that could be correlated to the materials implant site.

Results and Discussion

Study I – CombiCTx: screening diffusion gradients of anti-cancer drug combinations

In this study, we developed CombiCTx, a novel *in vitro* assay for combinatorial drug testing.

Main results and discussion

This study was inspired by the CombiANT assay for antibiotic interaction testing¹³⁸ and the main objective was to redesign this assay for combinatorial drug testing of anti-cancer drugs. Therefore, the first challenge was to adapt the CombiANT assay to the constraints of adherent mammalian cell culture and subsequently, develop an assay and imaging protocol for proof-of-concept drug synergy testing.

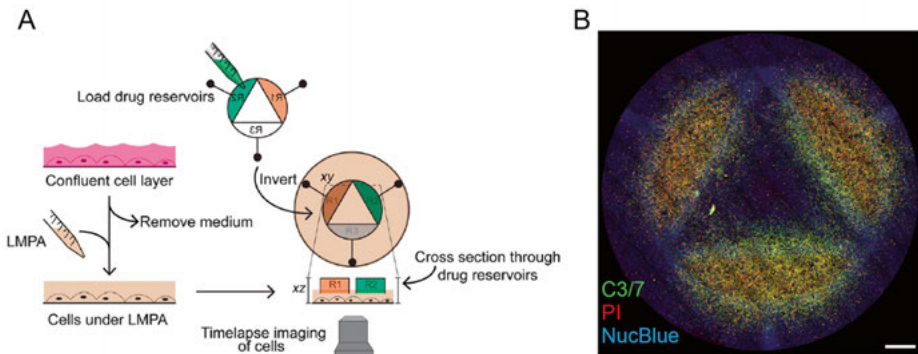


Figure 2. (A) Illustration of the CombiCTx assay workflow (B) Example of an assay read out with caspase 3/7, PI and NucBlue staining. Scale bar: 2 mm. From Study I, Stelzl et al., 2026

The CombiCTx device consists of three reservoirs, which are supported by support struts, around a central triangular area. At the beginning of the assay the reservoirs are filled with low melting point agarose (LMPA) in which the drugs of choice are dissolved. After polymerisation of the LMPA in the reservoirs, the CombiCTx insert can be inverted and carefully placed on top of a 2D layer of cells covered in, freshly poured unpolymerized LMPA.

Once this layer of LMPA is also polymerised, it forms a continuous gel layer that connects the downward facing drug reservoirs and the cells. This enables drug diffusion from the reservoirs to the cells and the formation of dynamic drug gradients.

Visualisation of these diffusion gradients was initially performed with fluorophores, fluorescein 5/6-isothiocyanate (FITC) and tetramethylrhodamine-5-isothiocyanate (TRITC), showing the overlapping diffusion gradients that form when two similar sized molecules diffuse through the assay space. Subsequently, doxorubicin (DOX), a clinically relevant chemotherapeutic, with fluorescent properties, was chosen to illustrate drug diffusion itself.

To assess the cell death and apoptosis response of breast cancer cells (MDA-MB-231), a double staining imaging protocol was implemented. For these initial drug response assays, we chose to use staurosporine. In previous work we had established staurosporine concentrations that induced apoptosis and cell death in 2D cultures of MDA-MB-231 cells ¹⁵⁰. An imaging protocol using an apoptosis (Caspase 3/7) and cell death (propidium iodide) double staining was implemented to assess the cell response of MDA-MB-231 cells.

Finally, the CombiCTx assay was applied to investigate the combinatorial effects of two anti-cancer drugs, navitoclax (Nav), a BCL-2 inhibitor, and gemcitabine (Gem), a nucleoside analogue that interferes with DNA synthesis ^{151,152}. These two drugs were previously identified to have a synergistic effect on MDA-MB-231 cells ⁹⁴, something we also observed in 2D tests. Interestingly, the CombiCTx assay also identified a potential mild synergistic effect; however, this was localized to specific areas in the assay space. More specifically, this was observed in the region of interest (ROI) directly under the Nav loaded reservoir in assays loaded with both Gem+Nav, indicating that the diffusion of Gem into this space lead to a higher degree of cell death in this region than if only Nav was present. Yet, this effect was not seen in the ROI in the centre of the assay space or directly under the Gem loaded reservoir. This indicates that Nav did not diffuse as readily across the assay area as Gem. Indeed, Nav is a bigger molecule and is more lipophilic with a higher protein binding affinity ^{153,154}. This shows that CombiCTx not only models one aspect of the tumour microenvironment, namely, diffusion through the ECM, but that this aspect can have important implications for understanding the effects of combinatorial drug treatments on cells.

Limitations and future perspectives

Screening for combinatorial drug effects is often performed in 2D assays, which enables high throughput analysis that can be partially automated, but these assays are limited in predicting the drug interactions within the TME⁹². In the CombiCTx assay, the diffusion of drugs through a 3D matrix was integrated to establish drug gradients as additional information beyond standard 2D assays. Yet, compared to the dynamic and complex TME, this is still a simplified model. Nevertheless, during method development of a novel 3D cell culture assay, the increase in complexity needs to be carefully balanced with the ability to troubleshoot and correctly interpret readouts. Therefore, certain limitations apply to the current CombiCTx assay, which should be addressed in future experiments.

The choice of matrix was governed by two major considerations. Firstly, the expected effect that matrix molecules would have on drug diffusion and on cell responses. It may appear counterintuitive to choose LMPA, which was expected to have very limited impact on both; however, this simplified expected diffusion pattern and cell response validation. In the next step of assay development, ECM molecules could be introduced for a more physiological cell environment, for example, by introducing biomimetic gels of different stiffness, which have been shown to impact drug diffusion^{155,156}. Secondly, LMPA has a low melting point and polymerises at room temperature, which contributes to the ease of handling during assay development. Nonetheless, the assay is still sensitive to environmental factors such as changes in ambient temperature. Overall, the established protocol is easy to perform, but user errors, such as introduction of bubbles through incorrect device placement, can also affect experimental outcome, highlighting the need for proper user training.

Additionally, to enable the incorporation into standard cell culture workflows, the cells are grown in a 2D layer on cell culture plastic. This also facilitated imaging of cell responses without the need for z-stack acquisition or more complex image analysis. However, in future studies, 3D cell culture modalities could be introduced by suspending cells within the gel layer or alternatively embedding spheroids, enabling increased cell-cell interaction.

Overall, the CombiCTx device and assay protocol offer a new way to study drug combinations, by incorporating drug diffusion in a 3D environment. This should now be validated using more drug combinations, and future assay iterations may further increase the complexity of this model.

Study II- An open source extrusion bioprinter based on the E3D motion system and tool changer to enable FRESH and multimaterial bioprinting

In this study, we developed an open source extrusion bioprinter built on the E3D motion system with a tool changer. The overall aim was to provide a cost-effective, user-friendly solution for high-resolution and multimaterial bioprinting.

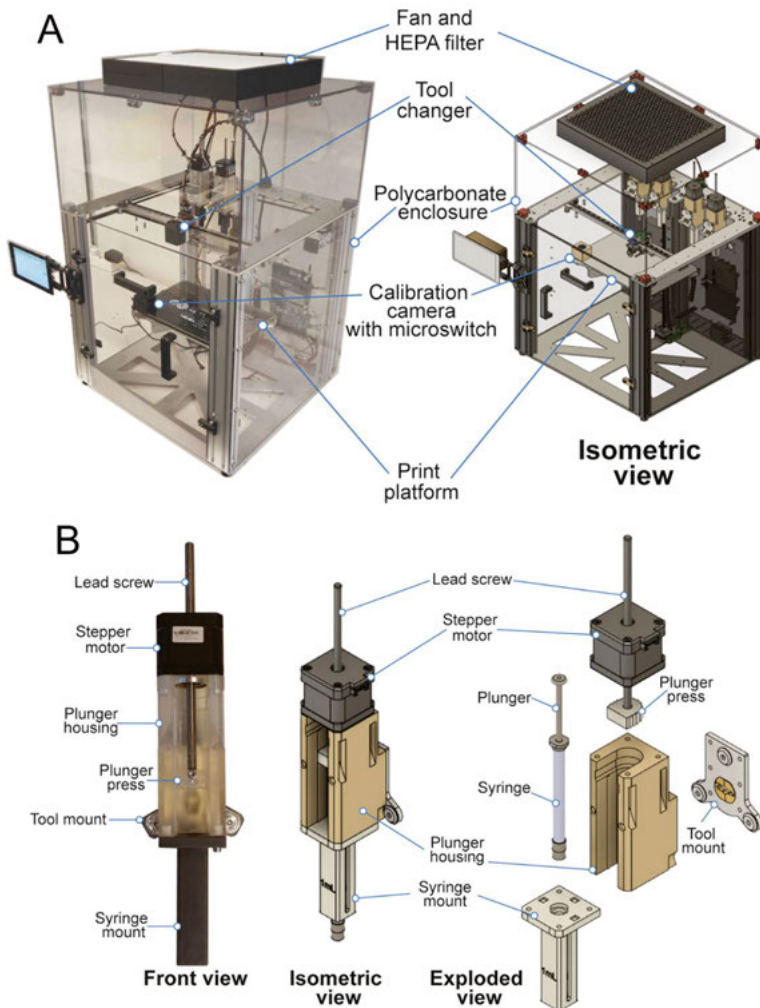


Figure 3. The open source bioprinter (A) Photograph (left) and CAD illustration (right) of the open source bioprinter. (B) Design of the syringe pump extrusion tool, photograph (left) and CAD design with exploded view (right). From Study II, Engberg et al. 2021

Main results and discussion

The bioprinter, as shown in Figure 3, was equipped with a custom-built syringe pump tool for extrusion bioprinting. The syringe pump, Figure 3B, is based on a stepper motor that drives a lead screw, which in turn presses on the syringe plunger and thus controls extrusion.

Shortly before this project was initiated, Lee et al. showed the versatility of the FRESH technique and its high resolution in collagen printing⁷¹. Thereafter it was quickly adopted by the bioprinting community. Thus, the establishment of a FRESH bioprinting protocol on our bioprinter was one of the objectives in this study.

To demonstrate the feasibility of FRESH printing, we used the commercially available LifeInk 240 (Advanced Biomatrix) and FRESH LifeSupport (Cellink) to print collagen scaffolds. MDA-MB-231 cells were subsequently seeded onto the printed scaffolds. The experiments showed robust cell attachment and viability, after overnight and one week of incubation, identified via live/dead staining. Additionally, the cell morphology was observed through actin staining in combination with a nuclear cell stain, showing normal cell morphology. In both experiments, we also observed a certain tendency of the cells to align along the printed collagen strands. A similar tendency was observed in Study IV when bone cells were seeded on the dense collagen ink. Cell adhesion to the LifeInk 240 was expected, as the final collagen scaffold consists of fibrillar collagen that MDA-MB-231 should readily attach to.

Successful printing of cell-laden bioink was also demonstrated with MDA-MB-231 cells encapsulated into a laminin bioink. Live/dead staining revealed a similar cell viability in bioprinted scaffolds and in gels extruded manually from a syringe, indicating that cell viability was not impacted by the printing process. However, the cells suspended in the bioprinted scaffolds had a rounded morphology. Such a change in cell morphology in response to culturing MDA-MB-231 cells has previously been reported¹⁵⁷, but is in contrast to other reports describing a stellate phenotype in 3D laminin-rich culture setups¹¹⁴. Another factor influencing cell morphology in 3D hydrogel cultures is stiffness and it has been shown that MDA-MB-231 cells exhibit different cell morphologies based on the hydrogel stiffness, with rounded cell morphologies in soft gels¹⁵⁸.

In conclusion, the open source bioprinter introduced in this study is a cost-effective and versatile bioprinter. We demonstrated the ability to use an

important, at the time of publication, recently published bioprinting technology as well as the capability of multimaterial printing.

Strengths and limitations

Since the publication of this study in 2021, this printer was adopted by several labs in Sweden and has become a standard tool in our lab. For instance, Kon-takis et al. used it to bioprint collagen models of trabecular bone¹⁵⁵. Extended use over several years has highlighted both the strength as well as limitations of the system.

The bioprinter remains an easy-to-use tool; however, it does require a certain understanding of g-code commands and the interaction between software and hardware. For example, this extrusion bioprinter is heavily based on FDM printing technology and most users adopt standard 3D printing slicing software to generate the g-code. Thus, the printing parameters need to be translated between bioprinting and FDM printing, e.g., filament thickness vs. inner syringe diameter. The advantage of the system, though, is full control over all printing parameters as well as access to all print files and settings. This is very crucial to facilitate troubleshooting, customisation as well as file sharing between users.

Bioprinting offers excellent opportunities for high-throughput production, which is also possible on our system, for instance using macros. One recurring issue is the adjustment of the z-axis, which is done at the beginning of each print to allow for optimal bioink flow. Standard plastic well plates, e.g., 96-well plates, were identified to have slight variations in the thickness of the bottom which can lead to suboptimal nozzle-substrate distance, which affects extrusion quality. To circumvent this, users may have to adjust the z-axis between each well or choose glass bottom dishes that have more consistent thickness.

The open source nature of the bioprinting system allows for the integration of new tools. Our group has worked on the establishment of a mixing tool that could easily change the composition of the extruded material during a print and allows for the generation of gradients (Moulin et al., manuscript under preparation). This would allow for the use of different materials within one print without changing the tool head.

Another strength is the cost-effectiveness of the bioprinter compared to commercially available printers with similar capabilities. However, by comparison commercial systems often require minimal background knowledge and have

integrated slicing abilities which may appeal to inexperienced users. Additionally, the availability of both custom-made and commercial platforms has increased since the publication of this study ^{156,157}.

Nonetheless, the open source bioprinter is a robust, customisable system that enables bioprinting of complex tissue models.

Study III – *In vivo* safety assessment of a bio-inspired bone adhesive & Study IV – Phosphoserine enriched dense collagen bioink

The novel bone adhesive, a phosphoserine modified calcium phosphate cement (PM-CPC), was proposed as a strong resorbable bone fixation strategy¹⁶². This project investigated the biosafety of this PM-CPC both *in vivo* and *in vitro*.

Main results study III

Six different calcium phosphate cement formulations were investigated as solid discs, four of which contained varying concentrations of phosphoserine (pSER) and silicate, while one control group contained only silicate and the final group was alpha-tricalcium phosphate (α -TCP) without any additional modifications.

In vitro results showed that all adhesive formulations had comparable cell viability to the α -TCP control; however, viability was significantly lower on the silicate-containing α -TCP control group. Calcium phosphate cements, including α -TCP, are used in clinical settings for bone fixation and other applications and are therefore considered biocompatible^{163,164}. Yet, when the CPC groups were compared to the cell viability of cells seeded on tissue culture plastic, the viability was moderate (45-64% for the adhesive groups). Thus, the reduced viability compared to tissue culture plastic may be partially due to differences in cell seeding or cell adhesion efficiency on the CPC surfaces. It should also be noted that the cells were seeded directly on the PM-CPC discs without any prior pH adjustment. Based on these results, we decided to control for pH differences in the subsequent study of the pSER modified collagen ink (Study IV).

Histological observation of subcutaneous implants in rats after 6- and 12-weeks of implantation, revealed no adverse tissue reaction. For further understanding of potential underlying mechanisms, gene expression analysis of inflammatory markers (IL1, IL6, TNF α) and bone marker genes (Colla1, SOX9, RUNX2) was performed. While no effect was observed in the soft tissue surrounding the implants, all examined genes were expressed at low levels with large variation. Due to the large variation and the fact that only one reference gene was used, these results should be interpreted with caution.

Overall, the results indicate no increase in inflammation or immune response and no ectopic bone formation, suggesting good biocompatibility in a preclinical setting, findings that have later been confirmed in functional studies¹⁶⁵.

The bone adhesive and other phosphoserine modified materials

Study III was the first study investigating the biosafety of this novel bone adhesive *in vivo*. Later, the adhesive properties and bone healing effects were proven in animal models¹⁶⁵. Based on the preclinical evaluations several patents were filed (US Patent App. 19/056,270, US Patent 12,251,490, US Patent App. 18/612,434, US Patent 11,964,072, US Patent 11,925,722). This adhesive is now a product, and it was recently (March 2026) announced that the Medicines and Healthcare Products Regulatory Agency in the UK has approved it for a first-in-human clinical investigation of tibial plateau fracture fixation. The study, led by Professor Peter Giannoudis, is being conducted at Leeds University Teaching Hospital.

The suitability of pSER as an osteoconductive material modification has previously been proposed by other groups¹⁶⁶⁻¹⁶⁹. pSER/HA composites, with or without collagen in the CPC, showed good cell viability, increased proliferation, increased osteoblast differentiation and higher expression of osteoblastic marker genes^{166,168}. Interestingly, CPC/collagen modification with pSER resulted in similar support of osteogenic differentiation of human bone marrow stromal cells (hBMSCs) as when modified with osteocalcin¹⁶⁸.

According to Park et al., pSER supplementation in the cell medium, even without additional biomaterials, increased matrix mineralisation and ALP activity, while pSER loaded hyaluronic acid hydrogels induced new bone formation *in vivo*¹⁷⁰. Furthermore, pSER collagen/nanohydroxyapatite scaffolds were shown to positively affect hBMC cell viability, human bone osteosarcoma cell (MG63) proliferation and lead to increased cell differentiation as indicated by ALP activity and gene expression analysis *in vitro*. Additionally, the material also showed favourable effects *in vivo*.

Altogether, these studies showed the potential of a single phosphorylated amino acid, pSER, as a biomaterial modification to influence cell behaviour *in vitro* and contribute to enhanced bone healing *in vivo*. However, the mechanism behind these effects is not yet elucidated. It is known, that several non-collagenous ECM proteins, including osteopontin, bone sialoprotein and osteocalcin, are rich in phosphorylated amino acids, including pSER^{171,172}. These proteins are known to regulate mineral nucleation and bone turnover. Furthermore, phosphorylated amino acids, such as pSER, form part of their calcium phosphate binding motifs^{100,171-173}. These highly phosphorylated proteins are also localised at bone fracture surfaces and may be implicated with fracture toughness^{174,175}. The positive effect on bone regeneration of bone sialoprotein

(BSP) was, for instance, shown through introducing BSP into collagen hydrogels¹⁷⁶. It is theorised that pSER may be enough to replicate the influence of these non-collagenous ECM proteins^{168,169}.

Based on these studies and the success of the pSER-based bone adhesive, we concluded that further investigation of pSER is warranted. Inspired by Salgado et al¹⁶⁹ we decided to investigate the effect of pSER and calcium phosphate in collagen hydrogels.

Main results and discussion study IV

Recently, a dense collagen-silica hybrid bioink (DCSi) was developed as a mechanically strong ECM-mimicking hydrogel and was shown to be biocompatible *in vitro* and *in vivo*⁶⁵. In Study IV we explored the effects of pSER modified DCSi scaffolds on the osteogenic responses *in vivo* as well as cell viability *in vitro*, and whether this is influenced by prior mineralisation of the DCSi scaffolds (MDCSi). Figure 4 shows an overview of the DCSi scaffold treatments. Mineralisation, according to the protocol described in the manuscript, leads to calcium phosphate deposition on these scaffolds as shown by Norein et al. (manuscript in preparation). Additionally, through testing pSER and nanohydroxyapatite (nHA) as cell culture supplements, the consequences of the pSER and calcium phosphate combination were studied without the additional influence of collagen in the 3D printed scaffolds. Thus, we investigated cell viability both in standard 2D culture as well as on cell-seeded 3D printed scaffolds.

In the standard 2D cell viability testing, preosteoblasts (MC3T3-E1) and mesenchymal stem cells (rMSCs) were treated with pSER, nHA, or the combination of both at varying concentrations for 3 days. A dose-dependent effect of the pSER/nHA combination was identified for MC3T3-E1 cells. Low concentrations of pSER/nHA led to an increase in cell viability, with the most pronounced effect at 0.1% and 0.05%, whereas a high concentration (1% pSER/nHA) resulted in a significant decrease in cell viability in comparison to the 0% cell control. Treatment with nHA alone increased cell viability at 0.1% nHA, but no significant effect was observed at the other concentrations. In contrast, MC3T3-E1 cells did not show a significant response to varying concentrations of pSER alone, which is in alignment with previously published findings¹⁷⁰.

Interestingly, rMSCs did not exhibit increased cell viability. Instead, a slight trend toward decreased cell viability was observed. However, as the data

presented for rMSCs are based on a single experimental repeat, these should be interpreted with caution.

Overall, the results suggest a dose-dependent and potentially cell type-dependent effect of low concentrations of pSER/nHA at the investigated time point. The observed increase in metabolic cell viability signal may reflect increased proliferation, which is also supported by the illustrative live/dead staining microscopy images, or alternatively reflect increased metabolic activity. Nevertheless, further experiments would be required to confirm this.

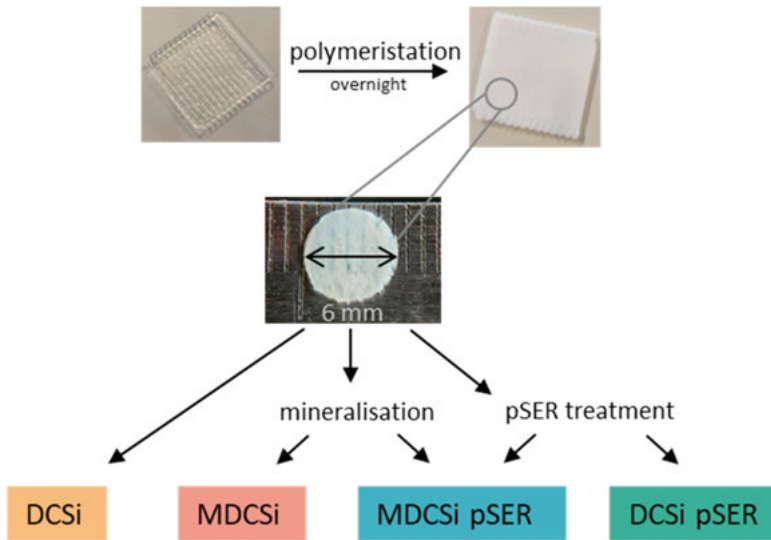


Figure 4. Overview of 3D bioprinted DCSi scaffold treatment. The top left shows the initial scaffold directly after printing, followed by overnight polymerisation (top right). The centre image shows an example of a final scaffold and arrows are indicating treatment groups. DCSi – untreated collagen scaffold; MDCSi – mineralised collagen scaffold; MDCSi pSER – mineralised scaffold with pSER treatment; and DCSi pSER – collagen scaffold with pSER treatment

Consistent with the 2D results, MC3T3-E1 cells seeded on mineralised pSER-modified collagen scaffolds (MDCSi pSER) showed a comparable dose-dependent response. In a screening of several pSER concentrations, an increased cell viability was observed at 0.025% pSER and 0.05% pSER on MDCSi scaffolds. Similarly to the 2D experiments, high concentrations of pSER were detrimental to cell health, with very low viability signal on MDCSi 5% pSER scaffolds. It is interesting that similar concentrations of pSER were identified as having a positive influence on cell viability in 2D and 3D, although the experimental setups appear fundamentally different. However, this indicates that in both culture models MC3T3-E1 cells were exposed to similar levels of pSER.

This suggests that the observed effect of pSER in combination with calcium phosphate (either through mineralisation of the DCSi scaffolds or as nHA particles in the cell medium) is not limited to the simplified 2D culture system but is also maintained in an ECM-mimicking 3D hydrogel environment.

To evaluate the osteogenic response to the pSER modified scaffolds *in vivo*, a unicortical defect model was chosen ¹⁴⁰. For this experiment the MDCSi pSER and DCSi pSER scaffolds were conditioned in 5% pSER. This concentration was based on previous studies showing that materials containing 5% pSER enhanced osteogenic response *in vivo* in porous collagen scaffolds ¹⁶⁹, as well as enhanced osteogenic differentiation of cells *in vitro* ¹⁶⁸. In the unicortical femoral defect model both groups of mineralised scaffolds (MDCSi pSER and MDCSi) showed increased bone volume fraction on μ CT analysis as well as a trend toward a pSER-related response. This corresponds with previously published studies reporting increased osteogenic effect in this type of material ¹⁶⁹.

However, we also tested the MDCSi 5% pSER scaffolds *in vitro*, where we observed a significant decrease in cell viability. This discrepancy between the *in vitro* and *in vivo* results as well as published data in the literature, including Study III, highlights the difficulty in comparing results across different experimental models. It could be expected that the effective availability of pSER is different depending on the experimental setup. Whether pSER is supplemented in the cell medium, incorporated into solid CPC discs, or adsorbed to scaffold surfaces may influence how cells are exposed to pSER. Consequently, the local concentration of pSER experienced by the cells may differ substantially between experimental models. In particular, possible diffusion of pSER from the collagen scaffolds could be problematic in a closed *in vitro* system lacking physiological functions to balance fluctuating pH or amino acid levels.

Altogether, the observations indicate that the combination of pSER with calcium phosphate has an osteogenic effect and that the observed effect on cell viability was dose-dependent *in vitro*.

Limitations and future perspectives

Based on our own results in Study III and IV, as well as the literature, it seems that pSER modified materials are strong candidates as osteoconductive biomaterials. Therefore, the data presented in Study IV are part of an ongoing project aiming to investigate the role of pSER as a stimulator of osteogenic responses.

In the *in vitro* assays, a first indication of cell-type-dependent response was detected. This should be further investigated by adding more independent repeats for the rMSC data, but also by investigating other cell types. Of special interest are primary cells, such as patient-derived human osteoblasts. Testing of additional bone cell lines on the MDCSi pSER scaffolds would also contribute to enhanced comparability with existing data in the literature. Additionally, a first repeat of a time-dependent cell viability assay of cells seeded on the collagen scaffolds indicated a time-dependent effect on MDCSi 0.05% pSER scaffolds. This should also be repeated to confirm the observation. In our study we currently focus on early time points (maximum seven days) in the *in vitro* experiments. Later time points may also be interesting to understand long-term effects, especially to compare our data to published literature. However, our focus on early time points was additionally based on discussions with collaborators including Christiane Salgado, author of one of the key publications for Study IV on pSER modified scaffolds¹⁶⁹. Differences in cell adhesion between the scaffold compositions could also be investigated to clarify how variations in surface morphology across the scaffold groups contribute to these differences.

Furthermore, the current *in vitro* investigation focused solely on cell viability assessment. To understand the true osteogenic potential of the MDCSi scaffolds, gene expression analysis of bone marker genes should be conducted. Several authors have reported an effect of pSER on bone cell differentiation^{166,168–170}. Additionally, functional assays such as alkaline phosphatase activity or matrix mineralisation could further clarify the osteogenic potential of the MDCSi scaffolds.

Overall, MDCSi scaffolds are promising candidates as resorbable material for bone regeneration. While the role of phosphorylated serine in combination with calcium phosphate in bone regeneration needs to be further studied, the data presented here provide further indication for its potential effect.

Conclusions

Additive manufacturing offers new possibilities to create more advanced cell culture models both through conventional 3D printing as well as bioprinting. In this thesis, the capabilities of additive manufacturing to help transition from standard 2D cell culture models to more advanced models were explored. It was shown how additive manufacturing can be used to build new tools and prototype cell culture devices. Additionally, different bioprinting approaches showed how these technologies can be used to study cell-biomaterial interactions.

Anti-cancer drug therapies are often based on combinatorial drug regimens, yet the identification of suitable drug combinations is difficult. In Study I, a novel anti-cancer drug testing device, CombiCTx, was developed. A protocol for combinatorial drug testing and image analysis was proposed and it was shown that synergies can be successfully identified. Overall, the CombiCTx assay provides a new way of studying drug combinations by incorporating drug diffusion through a gel. Therefore, the device enables the mimicking of some aspects of the complex tumour microenvironment.

The accessibility to bioprinting has been partially limited by availability and cost of bioprinting systems. Therefore, in Study II an open source bioprinter capable of multimaterial and high resolution bioprinting was developed. The successful bioprinting of cell-laden bioinks and the establishment of FRESH bioprinting protocol on the system were demonstrated. The open source bioprinter presents a customisable and cost-effective platform to bioprint for tissue modelling or drug screening applications.

Resorbable osteoconductive materials are being investigated as strategies to improve bone healing. In Study III, an *in vitro* and *in vivo* biosafety assessment of a novel bone adhesive based on pSER-modified calcium phosphate cements was performed. No adverse tissue reactions were observed in a subcutaneous rat model and *in vitro* cell viability testing showed similar biocompatibility compared to the control group, demonstrating the safety of the bone adhesive in a preclinical setting. While this study used standard 2D biomaterials testing, the concept of pSER-modified materials for bone tissue

regeneration was revisited in Study IV. Here the effect of pSER treatment and mineralisation of collagen scaffolds was investigated. Both standard 2D cell viability testing and evaluation of cell viability in the scaffolds showed a dose-dependent effect of pSER in combination with calcium phosphate. An osteogenic effect of the MDCSi pSER scaffolds was also demonstrated *in vivo*. Together, these findings suggest the potential of pSER-modified materials for bone regeneration.

Overall, the work presented in this thesis explored some of the advantages and limitations of additive manufacturing for the development of advanced cell models. The presented studies provide potential to be further developed and explored for more complex *in vitro* models. Continued development of the CombiCTx assay, for instance by incorporating physiologically relevant ECM molecules or adding 3D cell culture capabilities, could further refine the method. The open source nature of the bioprinter enables new tools to be added, which is currently being explored in ongoing research in our group, to further increase the achievable complexity of bioprinted tissue models. Cellular responses to MDCSi pSER scaffolds should be further investigated by gene expression and differentiation studies, which may contribute to understanding the mechanisms behind the pSER induced bone healing potential.

Finally, these studies are parts of many ongoing research projects aiming to improve the predictive value of *in vitro* models with the goal to reduce the number of necessary *in vivo* studies. While this thesis only investigated selected aspects of additive manufacturing, it contributes a small piece of the larger effort of developing advanced *in vitro* models that may ultimately improve the translation of preclinical research into clinical applications.

Popular science summary

To study new drugs or biomaterials before they are used in patients, scientists often rely on simplified cell models in the laboratory. Most commonly, cells are grown as thin layers on flat plastic surfaces, known as 2D cell culture. While this approach has contributed to many important discoveries, it does not reflect the complex three-dimensional environment that cells experience in the human body. As a result, promising findings in the lab do not always translate into effective treatments for patients.

To bridge this gap, and to reduce the need for animal experiments, there is a growing need for more complex cell culture models. In this thesis, we explored how 3D printing technologies can be used to move from simple 2D systems toward more advanced 3D models for drug testing and biomaterials research.

In cancer therapy, patients often receive combinations of drugs, but identifying new effective combinations remains challenging. To address this problem, in Study I we developed a new cell culture device, called CombiCTx to test combinations of anti-cancer drugs. Instead of exposing cells to uniform drug concentrations, this system creates gradients where drugs diffuse through a gel, more closely resembling how drugs spread within a tumour. We showed that this approach can be used to identify two drugs that work better together than when used alone. However, this effect was localised to a small area of the assay, highlighting that the way drugs diffuse is an important factor that is not accounted for in standard cell experiments but may play a role within a tumour.

In the second study, we addressed a practical challenge: access to advanced bioprinting technology. We developed an open source bioprinter that is affordable and flexible. This printer was used to create collagen scaffolds that mimic the natural environment of cells and support cell growth. We were also able to use the bioprinter to print living cells in gels and keep the resulting cell-containing scaffolds viable. Using this system, we showed that it is

possible to reliably print structured biological materials and grow cells in a more realistic 3D environment.

The third and fourth studies focused on biomaterials for bone repair. We investigated a new bone adhesive inspired by natural bone chemistry, designed to act as a “glue” for fixing bone fragments in complex fractures. In cell culture experiments and animal studies, this material showed good biocompatibility and did not trigger harmful immune responses.

Building on this, we used 3D bioprinting to create collagen-based scaffolds modified with phosphoserine, an amino acid found in natural bone proteins. These scaffolds supported cell growth and showed signs of promoting bone formation in animal models. Interestingly, we also found that the effects of this material depended strongly on concentration and experimental conditions, highlighting the importance of testing materials in both simple and more advanced models.

Together, the work in this thesis demonstrates how additive manufacturing can be used to create more realistic, complex cell culture systems and to study new biomaterials. These approaches contribute to the development of better experimental models that may improve the prediction of treatment outcomes and ultimately support the development of new therapies.

Populärwissenschaftliche Zusammenfassung

Um neue Medikamente oder Biomaterialien zu untersuchen, bevor sie bei Patient:innen eingesetzt werden, greifen Wissenschaftler:innen häufig auf vereinfachte Zellmodelle im Labor zurück. Meist werden Zellen dabei als dünne Schichten auf flachen Kunststoffoberflächen kultiviert, sogenannte 2D-Zellkulturen. Obwohl dieser Ansatz zu vielen wichtigen Entdeckungen beigetragen hat, spiegelt er nicht die komplexe dreidimensionale Umgebung wider, der Zellen im menschlichen Körper ausgesetzt sind. Daher lassen sich vielversprechende Ergebnisse aus dem Labor nicht immer direkt in wirksame Therapien für Patient:innen übertragen.

Um diese Lücke zu schließen und gleichzeitig den Bedarf an Tierversuchen zu verringern, werden zunehmend komplexere Zellkulturmodelle benötigt. In dieser Arbeit wurde untersucht, wie 3D-Drucktechnologien genutzt werden können, um von einfachen 2D-Systemen zu fortschrittlicheren 3D-Modellen für die Wirkstoffforschung und die Untersuchung von Biomaterialien zu gelangen.

In der Krebstherapie erhalten Patient:innen häufig Kombinationen verschiedener Medikamente, doch die Identifizierung neuer wirksamer Kombinationen ist weiterhin eine Herausforderung. Um dieses Problem zu adressieren, entwickelten wir in der ersten Studie ein neues Zellkultursystem namens CombiCTx, mit dem sich Kombinationen von Krebsmedikamenten untersuchen lassen. Anstatt Zellen gleichmäßigen Wirkstoffkonzentrationen auszusetzen, erzeugt dieses System Konzentrationsgradienten, bei denen sich die Wirkstoffe durch ein Gel ausbreiten, ähnlich wie es auch in einem Tumor geschieht. Wir konnten zeigen, dass sich mit diesem Ansatz zwei Wirkstoffe identifizieren lassen, die in Kombination besser wirken als einzeln. Die Tatsache, dass dieser Effekt auf einen kleinen Bereich des Systems begrenzt war, weist darauf hin, dass die Art und Weise, wie sich Wirkstoffe ausbreiten, ein wichtiger Faktor ist, der in Standardexperimenten oft übersehen wird, im Tumor jedoch eine Rolle spielen kann.

In der zweiten Studie beschäftigten wir uns mit einer praktischen Herausforderung: dem Zugang zu moderner Bioprinting-Technologie. Wir entwickelten einen Open Source Bioprinter, der kostengünstig und flexibel einsetzbar ist. Mit diesem Drucker konnten wir Kollagengerüste herstellen, die die natürliche Umgebung von Zellen nachahmen und deren Wachstum unterstützen. Zudem gelang es uns, lebende Zellen in Gelen zu drucken und die so erzeugten zellhaltigen Strukturen lebensfähig zu halten. Wir konnten zeigen, dass sich mit diesem System strukturierte biologische Materialien zuverlässig herstellen und Zellen in einer realistischeren dreidimensionalen Umgebung kultivieren lassen.

Die dritte und vierte Studie konzentrierten sich auf Biomaterialien für die Knochenheilung. Wir untersuchten einen neuartigen Knochenklebstoff, der von der natürlichen Zusammensetzung des Knochens inspiriert ist und dazu entwickelt wurde, Knochenfragmente bei komplexen Frakturen wie ein „Klebstoff“ zu fixieren. In Zellkultur- und Tierexperimenten zeigte dieses Material eine gute Biokompatibilität und löste keine schädlichen Immunreaktionen aus.

Darauf aufbauend nutzten wir 3D-Bioprinting, um kollagenbasierte Gerüste herzustellen, die mit Phosphoserin, einer Aminosäure, die in natürlichen Knochenproteinen vorkommt, modifiziert wurden. Diese Gerüste unterstützten das Zellwachstum und zeigten in Tiermodellen Anzeichen einer verbesserten Knochenbildung. Interessanterweise stellten wir außerdem fest, dass die Wirkung dieses Materials stark von der Konzentration und den experimentellen Bedingungen abhängt, was die Bedeutung von Tests sowohl in einfachen als auch in komplexeren Modellsystemen unterstreicht.

Insgesamt zeigt diese Arbeit, wie 3D-Druck genutzt werden kann, um realistischere und komplexere Zellkultursysteme zu entwickeln und neue Biomaterialien zu untersuchen. Diese Ansätze tragen zur Entwicklung besserer experimenteller Modelle bei, die die Vorhersage von Behandlungsergebnissen verbessern und letztlich die Entwicklung neuer Therapien unterstützen können.

Disclosure of AI use

During the preparation of this work Open AI's Chat GPT-5.3 was used for language editing and proofreading. I carefully reviewed and edited all suggestions and take full responsibility for the content of the publication.

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