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Advances in biofabrication techniques for collagen-based 3D *in vitro* culture models for breast cancer research

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ABSTRACT

Collagen is the most abundant component of the extracellular matrix (ECM), therefore it represents an ideal biomaterial for the culture of a variety of cell types. Recently, collagen-based scaffolds have shown promise as 3D culture platforms for breast cancer-based research. Two-dimensional (2D) *in vitro* culture models, while useful for gaining preliminary insights, are ultimately flawed as they do not adequately replicate the tumour microenvironment. As a result, they do not facilitate proper 3D cell-cell/cell-matrix interactions and often an exaggerated response to therapeutic agents occurs. The ECM plays a crucial role in the development and spread of cancer. Alterations within the ECM have a significant impact on the pathogenesis of cancer, the initiation of metastasis and ultimate progression of the disease. 3D *in vitro* culture models that aim to replicate the tumour microenvironment have the potential to offer a new frontier for cancer research with cell growth, morphology and genetic properties that more closely match *in vivo* cancers. While initial 3D *in vitro* culture models used in breast cancer research consisted of simple hydrogel platforms, recent advances in biofabrication techniques, including freeze-drying, electrospinning and 3D bioprinting, have enabled the fabrication of biomimetic collagen-based platforms that more closely replicate the breast cancer ECM. This review highlights the current application of collagen-based scaffolds as 3D *in vitro* culture models for breast cancer research, specifically for adherence-based scaffolds (*i.e.* matrix-assisted). Finally, the future perspectives of 3D *in vitro* breast cancer models and their potential to lead to an improved understanding of breast cancer diagnosis and treatment are discussed.

1. Introduction

Breast cancer is a major healthcare burden worldwide, with 2018 global estimates indicating the disease is responsible for 11.6% (~2.1 million) of all new cancer cases and the 5th highest cause of cancer mortality with 626,679 deaths (6.6% of total deaths) across both sexes [1]. Within the female population only, breast cancer is responsible for the highest incidence and mortality rates, accounting for 24.2% of all new cases and 15% of cancer deaths [1]. The 2018 data represents an increase compared to previous GLOBOCAN data of 1.7 million new cases and 522,00 deaths in 2012 [2]. With ageing populations, poorer diets and a host of other genetic and environmental factors, urgent action is needed to address breast cancer – particularly in the case of metastatic breast cancer where median overall survival remains poor at only 2–3

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years [3]. It is imperative to continue to invest in novel and alternative approaches to study breast cancer across many disciplines including cancer morphology, genetics, drug testing and more. 3D *in vitro* culture models are steadily emerging as leading research and developmental tools in the field of breast cancer research. These models overcome issues that are associated with traditional 2D *in vitro* culture and allow researchers the opportunity to gain more valuable insights into how different cancer types develop, progress and respond to various clinical regimes.

Many natural biomaterials have been used in the fabrication of 3D platforms for breast cancer culture, including collagen [4–7], fibroin [8–10], alginate [11–13], gelatin [13–15] and hyaluronic acid [16–18]. Collagen represents a very attractive option as it is the most abundant human protein (~30% of total body protein) and provides an essential structural role within connective tissue and the extracellular matrix (ECM). At a cellular level it is involved in cell adhesion, cell-cell and cell-matrix communication. As the principal component of ECM tissue, collagen plays a significant role in the tumour microenvironment [19–22] – notably so in breast cancer [23–25]. As a result, many collagen-based *in vitro* breast cancer culture models have been developed [4–7,26,27]. While initial studies used simple hydrogels, recent advances in biofabrication techniques provides opportunities to fabricate 3D breast cancer models that more closely replicate the tumour microenvironment. These biomimetic 3D breast cancer models will enable researchers to gain a deeper understanding of cancer development and progression, in addition to providing more biologically relevant platforms for testing the efficacy of traditional and novel cancer drug compounds. Use of 3D *in vitro* culture models can reduce the reliance on traditional 2D culture and potentially pave the way to reduce the use of animals in drug testing regimes, a key goal of the biopharmaceutical and medical device technology industries.

2. Emergence of 3D culture models

2D and 3D *in vitro* culture models are used to study many cancer types, both in terms of tumour morphology/characteristics and tumour response to therapeutic agents. The application of 3D *in vitro* culture models play a valuable role in cancer research, however there is currently no one validated, trusted model for breast cancer. As a result, 2D *in vitro* and *in vivo* (xenografts) culture remain popular and widely used [28]. 2D *in vitro* culture models involve the growth of cancer cell lines or dissociated primary tumour cells in a monolayer [29]. The use of primary cells is preferred as they match the original tumour [30]. However, culture lifespan of primary cells is limited and they are more difficult to grow [31,32]. As a result, cancer cell lines have been developed and have proven to be a useful resource in cancer research, however their use is not without limitation. Cell lines are robust and can replicate indefinitely – but they are clonally derived and thus homogeneous populations [33,34]. They do not replicate the heterogeneity displayed within tumours, an aspect of their morphology that makes certain cancers challenging to treat [35]. Certain cancer cell lines may have subtle genetic and epigenetic differences from primary counterparts, which may further reduce their usefulness in various *in vitro* experiments [36,37]. Furthermore, existing 2D *in vitro* culture methods do not adequately replicate the complex tumour microenvironment. The tumour-ECM interaction is fundamental in directing and controlling many aspects of cancer development and progression [20,22] and is absent in 2D culture models. The lack of the 3D matrix in 2D models results in poor cell-cell interaction and a lack of cell-matrix interactions. Also, there is a lack of nutrient/oxygen gradients in 2D culture, which is not replicative of human cancer [38]. A further consequence of these 2D culture models is altered/inflated drug response by cells grown in a monolayer [39–43]. Cells in their native 3D environment, coupled with the presence of extensive ECM, frequently have a higher resistance to various drug compounds than cell line counterparts in 2D culture. This can be seen in many 2D *versus* 3D studies, whereby different responses to drug exposure, not just limited to increased resistance, occurs in 3D culture models compared to the same cells grown in monolayer [39–43]. A large percentage of drugs that show promise in 2D *in vitro* culture models are not successful in clinical trials, with only a small percentage (<5%) progressing to the marketplace [44]. The primary advantages of 3D models over 2D models are highlighted in Fig. 1.

In vivo xenograft animal studies overcome many limitations of existing 2D and 3D models, however they also have associated challenges. The use of human cells in animal models has a range of potential limitations, including; size difference between humans and animals, slight genetic differences, differences in tumour microenvironment, non-orthotopic tumour development, and many xenografts are developed in immunocompromised animals meaning they lack the important interplay between tumours and the immune system [45]. These limitations contribute to the poor translation of results from preclinical animal trials to human trials [46,47]. Furthermore, increasing ethical consideration for animal welfare has led to calls to radically alter research practices, shining a light once more on the decades-old 'Three Rs' – to replace, reduce and refine our usage of animals in scientific research [48].

Incorporating the third dimension into *in vitro* settings for cancer-based research offers many potential advantages, *e.g.* the provision of stroma/matrix and thus cell-matrix interactions; improved resemblance of cells to *in vivo* counterparts in terms of shape, cell-cell interactions, behaviours and genetic profiles; development of heterogenous cell populations; co-culture of multiple cell types; variable access for cells to nutrients/oxygen as is the case in *in vivo* tumours; and a more clinically representative response to therapeutic agents as per solid tumours [29,49,50]. The inclusion of immune function to 3D models also offers significant potential due to the crucial role the immune system plays in cancer, both in terms of disease initiation and progression but also regards to therapeutic strategies [51,52]. There are limited examples of co-culture of breast cancer cells and immune cells to date [53–55], emphasising this type of co-culture is still in a nascent stage and that more research is needed to develop such complex models.

Spheroids were an initial 3D culture system and remain popular today [56–58]. They are scaffold/matrix-free 3D culture systems consisting of cellular colonies. Spheroids are an attractive platform due to low cost of fabrication and the self-assembly of the 3D structures. The 3D cell spheroids formed had advantages over 2D monolayer cultures, including but not limited to; the provision of cell-cell interactions, reproduced 3D morphology of tumours *in vitro*, the secretion of ECM components resulting in provision of cell-matrix interactions, development of oxygen, nutrient and waste gradients (such a feature is common of *in vivo* tumours). However, despite the biological advances of spheroids, they still had limitations, most importantly of which is the lack of extensive 3D ECM structures (*i.e.* a scaffold system) resulting in poor replication of the tumour-ECM interplay [59–61]. An advancement on spheroids in recent times has been the development of organoids. These are 3D aggregates of increased complexity composed of organ-specific stem cells or progenitor cells. They self-assemble into 3D aggregates upon introduction to a scaffold support [62–64], though scaffold free organoids are also possible [65,66]. Upon stem cell differentiation and proliferation, they generate *in vivo*-like structures with good resemblance to the parent organ *in vitro* [62–64]. They are frequently used in cancer research for disease modelling and drug screening and development. Advantages of organoid cultures include development of complex organ-like structures that closely resemble *in vivo* tissue both histologically and genetically, development of human-derived organoids allowing for personalised strategies, provision of good cell-cell and cell-matrix interactions, and relative ease and robustness of organoid development and maintenance. Some limitations include reproducibility concerns and high levels of heterogeneity, lack of validated models and established protocols, increased costs compared to other 3D models or 2D culture, and still may lack full replication of the complex tumour microenvironment thus tumour-stroma interactions remain limited

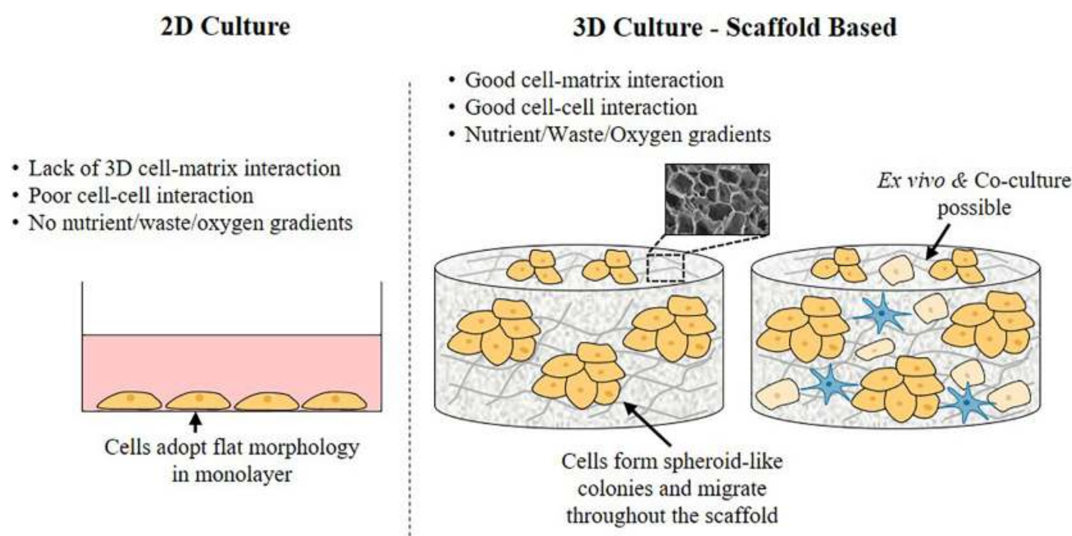

Fig. 1. 2D Culture comparison with 3D scaffold-based culture – This figure highlights some of the advantages of 3D scaffold culture over 2D culture. 3D scaffold culture provides the cell-matrix interactions and vastly superior cell-cell interactions that are not present in 2D culture. Co-culture with multiple cell types is also achievable using 3D scaffolds, which in a cancer setting is crucial as multiple cell types play a role in tumour progression through cell-signalling crosstalk. *Ex vivo* culture of patient cells is also achievable. Cells adopt a flat morphology in 2D monolayers, which facilitates an equal distribution of nutrients and oxygen. In 3D scaffolds, cells can form colonies that resemble tumour masses, which can generate internal gradients of nutrients and oxygen as is the case *in vivo* [38].

[67–69]. Many breast cancer organoids have been developed to date and been used to investigate breast cancer morphology, cell behaviours and drug discovery and testing [67,70–75]. While still an emerging and evolving area of research, organoids play a valuable role in cancer research.

Other platforms for 3D *in vitro* breast cancer models developed to date consist of, decellularised tissue structures [76–78], 3D scaffolds [4,5,7], hydrogels [27,79,80] and microfluidic devices [81–83]. This review will focus on matrix/adherent based systems such as scaffolds and hydrogels. These 3D structures will be referred to as '3D scaffolds' throughout this review. Many different materials have been used to develop 3D *in vitro* breast cancer scaffolds, generally categorised into naturally derived biomaterials (such as collagen [4–7], fibroin [8–10], alginate [11–13] and hyaluronic acid [16–18]) and synthetic polymers (such as polyethylene glycol (PEG) [84–86], poly(lactide-*co*-glycolide) (PLG) [87] or poly(ε-caprolactone) (PCL) [88–90]. While the successful culture of breast cancer cells has occurred using synthetic models [84–90], efforts have increased in the fabrication of biocompatible systems that more closely replicate the native tumour microenvironment. Replication of the tumour microenvironment is essential to bridge the gap between *in vitro* models and the *in vivo* environment and thus the ideal scaffold should contain biological components. As the primary component of ECM surrounding tumours, the use of collagen in the fabrication of 3D culture scaffolds is predominant and thus will be the primary focus of this review.

3. Extracellular matrix (ECM): collagens role in breast cancer

The ECM is a dynamic network of proteins that plays a role in tissue organisation, homeostasis maintenance and is also known for its preventative role in disease. Collagen is the most abundant protein present within the breast ECM, with laminins, elastin, fibronectin, proteoglycans, glycoproteins and a range of ECM remodelling enzymes also present [24]. Around the body, the ECM provides essential structural, biochemical and biomechanical support to cells [91,92]. The ECM also plays a key role in general cell activities including adhesion, proliferation, cell-cell communication and cell death [93,94]. It is through careful regulation of these processes that the ECM maintains a healthy and disease-free tissue state. However, recently the role that the ECM plays in carcinogenesis, metastatic spread and resistance to therapy has become more established, especially for breast cancer. Various alterations in the breast ECM structure, composition and component density (Fig. 2) have been highlighted as key occurrences in tumour growth, spread and resistance to treatments [20,24,95–98]. Collagen is the major component of the breast ECM and thus naturally plays a key role in the development and spread of cancer [23–25]. The collagen protein family, consisting of 28 known types [99], share a repeating amino acid sequence of Glycine-X-Y (where X and Y are other amino acids though frequently proline and hydroxyproline), with these repeating glycines at every third residue forming a triple helix collagen structure [99–101]. Collagen molecules interact with one another to form fibrils and these fibrils then further organise with other fibrils to form collagen fibres [100,102]. Depending on tissue type, collagen fibrils can organise and associate with one another in a varied manner to dictate specific tissue functions and properties [103]. Collagen has a critical role in aiding the structure and integrity of tissues and is a key player in cell signalling, differentiation and migration through cell-matrix interactions [100,101].

In breast cancer, increased deposition of collagen types I, III and V has been observed, which promotes tissue stiffness and is associated with increased cancer aggression and metastases risk [24,96,97,104]. Within the molecular/immunohistochemical (IHC) breast cancer subtypes there is emerging evidence of a correlation between subtype and collagen content/arrangement. Specifically, the IHC breast cancer subtypes include the following: Luminal A and B, human epidermal growth factor receptor-2 (HER2) enriched, and Basal-like (primarily consisting of triple negative breast cancers (TNBC)). Acerbi et al. (2015) found increased collagen deposition and fibre linearisation in the more aggressive HER2+ and TNBC tumours in comparison to both the less aggressive Luminal A and B subtypes [25]. The breast ECM is further stiffened through the action of lysyl oxidase (LOX) enzymes. These enzymes promote crosslinking of collagen within the ECM [105] and elevated LOX levels in breast cancer patients are associated with poor overall survival [19,106,107]. LOX expression has been observed to be significantly increased in triple negative breast cancers (advanced and aggressive disease) in comparison to the other subtypes [108]. Increased collagen expression and deposition are not the only alterations observed – the breakdown of collagen type IV is a crucial step in breast cancer metastasis [109]. Collagen type IV is a key component of the basement membrane [110] and its degradation is key to membrane breaching,

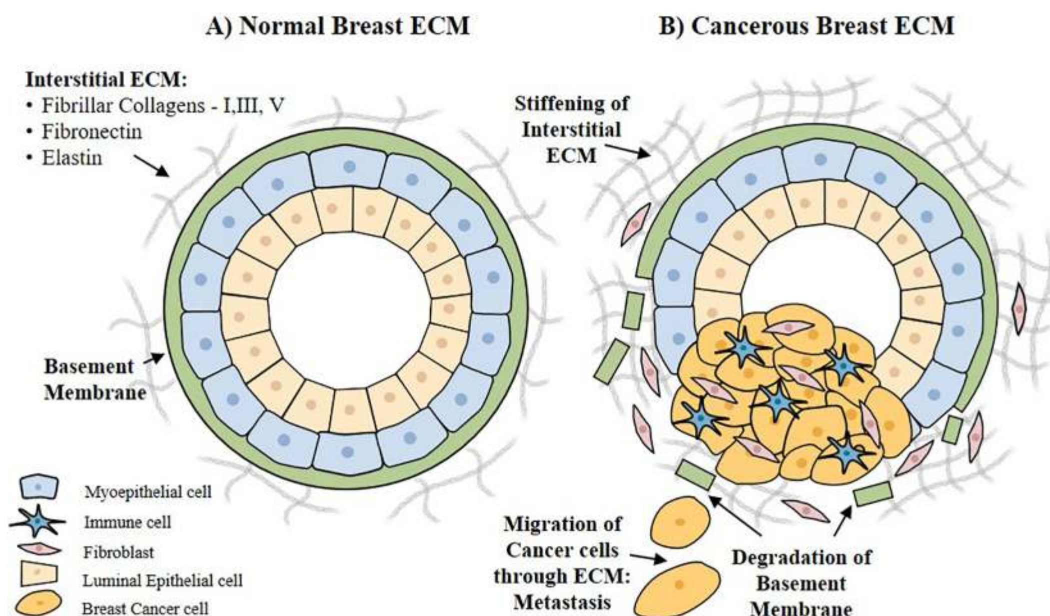

Fig. 2. Alterations in Breast ECM during cancer progression. This figure highlights the development of invasive Ductal Carcinoma, the most common form of breast cancer, and the key changes between the A) Normal Breast ECM and B) Cancerous Breast ECM. Key developments include the uncontrolled growth of tumour cells, disruption of the basement membrane, stiffening of the interstitial ECM and migration of cancer cells through the ECM. Significant alterations occur in the collagen makeup, namely the breakdown of the basement membrane and the increased deposition of collagen in the interstitial matrix.

allowing cancer cells to migrate to distant sites/organs. This degradation is suggested to be mediated by matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9 [111–114]. A further key signature of collagen alteration during breast cancer is the linearisation of collagen fibrils during carcinogenesis, which creates so-called ‘highways’ that facilitate the migration of cells away from the primary tumour site towards the basement membrane [98,115,116]. This feature is again seen to a greater extent in the more aggressive HER2+ and TNBC subtypes in comparison to the Luminal subtypes [25]. The stiffness of the ECM is correlated to the elastic modulus of tissue – stiffer tissues demonstrate increased elastic moduli and therefore leads to altered mechanical and biomechanical responses of the tissue, which often favours malignancy and cancer progression [19. 117]. There is limited agreement within the literature regarding the exact elastic modulus of the breast ECM. This is due to the difficulty in determining the mechanical properties of *in vivo* breast tissue and the variation in stiffness within different regions of the tumour microenvironment. Techniques used to measure tissue stiffness include standard unconfined compression, atomic force microscopy or elastography [19,118–123]. Healthy human breast tissue biopsies were found to have an elastic modulus ranging from ~1.00–1.83 kPa as determined using indentation-type atomic force microscopy. In contrast, cancerous breast tissue (patient IHC subtype not provided) demonstrates a large variance in stiffness with distinct soft regions in the tumour core of 0.3–0.75 kPa (densely populated with breast cancer cells) and significantly stiffer regions ranging from 2 to 20 kPa towards the tumour periphery (high collagen content) [118]. Atomic force microscopy analysis of breast ECM surrounding induced mammary tumours in an *in vivo* murine model (MMTV-PyMT mouse model, aggressive tumour resembling HER2+ subtype) exhibited an average elastic modulus of 1.1 kPa in normal pre-cancer ECM, increasing to 1.3 kPa in the pre-malignant state and further rising to 1.7 kPa in the malignant state [119]. In two other breast cancer murine model studies (both by unconfined compression): Paszek et al. found mean healthy breast tissue stiffness to be ~0.15–0.2 kPa, a mean tumoral tissue stiffness of ~3–5 kPa and adjacent breast tumoural tissue to have a mean stiffness of ~0.65–1.2 kPa (FVB-TgN (MMTV-c-myc, HER2/neu, and H-ras) – HER2+ subtype model) [120]; and Levental et al. found mean healthy breast tissue stiffness to be ~0.2 kPa, a mean tumoral tissue stiffness of ~1.25–1.75 kPa and adjacent breast tumoural tissue to have a mean stiffness of ~0.3–0.6 kPa (FVB-TgN MMTV-Neu mouse model – HER2+ subtype model) [19]. Tissue stiffness values determined using atomic force microscopy/compression testing can differ significantly to other techniques. Cancerous breast tissue stiffness measured through shear-wave elastography reported values ≥100 kPa (all subtypes, Luminal A/B, HER2 enriched and Basal-like (TNBC), assessed in each study) [121–123]. Thus, evidence in the literature suggests that the mechanical properties of breast cancer tissue are currently not yet fully understood, with few studies exploring the relationship between mechanical properties of the different breast cancer subtypes and how they may differ. It must also be noted that the complexity of human breast tissue is difficult to replicate in simple collagen-based 3D *in vitro* scaffolds. Tumour cells among other ECM components and cell groups all contribute to the mechanical properties of breast tissue *in vivo*.

The previously discussed correlation between breast cancer subtype and collagen deposition/arrangement [25] further translated into alignment of stiffness profiles with the various subtypes. Higher stiffness values were reported for more aggressive Basal-like (triple negative tumours) and HER2+ cancers (human samples), which had increased collagen deposition while the less aggressive Luminal A and B cancers showed a lower stiffness profile. In stiffness distribution profiles, determined through AFM, Basal-like and HER2+ had a large range of stiffness values with a greater skew towards stiffness values >2 kPa, with many samples seeing stiffness values of 1–6 kPa. Both Luminal A and B tumours had a smaller distribution of stiffness with the majority of samples in a range of 0.1–1.5 kPa [25]. These findings are summarised in Table 1. Similarly, Min Chang et al. (2013), through shear wave elastography, found HER2+ (160.3 kPa) and TNBC (165.8 kPa) tumours to have a greater mean stiffness than Luminal tumours (136.9 kPa) [122]. This potential correlation between collagen properties and breast cancer subtype may present the opportunity for stratification of *in vitro* work whereby 3D scaffolds are tailored based on the presenting patients breast cancer subtype.

Table 1
Breast Cancer environment by subtype and 3D scaffold properties required to support each subtypes growth.

IHC-Subtype	Characteristics	ECM properties	Suitable scaffold properties
Luminal A ER+ and/or PR+, HER2- [124,125], Ki-67 low [126,127]	The most prevalent subtype of breast cancer. Rather slow-growing tumours and have the best overall survival of all breast cancers. Non-aggressive with slow disease progression [128,129].	Luminal A tumours appear to have a low stiffness profile in the ECM/Stroma (0.1–1.5 kPa [25]). Collagen deposition during cancer progression is reduced in comparison to more aggressive subtypes HER2+ and Basal-like. Low linearisation of collagen fibres in comparison to HER2+ and TNBC subtypes [25].	Design of 3D scaffolds to mimic the ECM for culture of Luminal A cell lines (*e.g.* MCF7 [130])/primary cells requires a lower stiffness profile (0.1–1.5 kPa [25]) than HER2+ or TNBCs. Collagen fibres can be of random alignment (isotropic structure).
Luminal B ER+ and/or PR+, HER2- or HER2+ [124,125], Ki-67 high [126,127]	Luminal B is similar to Luminal A but has a more aggressive phenotype. Tumours proliferate at a higher rate than Luminal A and have a poorer prognosis [128,131,132].	Luminal B tumours appear to have a low stiffness profile in the ECM/stroma (0.1–1.5 kPa [25]). Collagen deposition during cancer progression is reduced in comparison to more aggressive subtypes HER2+ and Basal-like. Low linearisation of collagen fibres in comparison to HER2+ and TNBC subtypes [25].	Design of 3D scaffolds to mimic the ECM for culture of Luminal B cell lines (*e.g.* ZR7530 [130])/ Luminal B primary cells require a lower stiffness profile (0.1–1.5 kPa [25]) than HER2+ or TNBCs. Collagen fibres can be of random alignment (isotropic structure).
HER2 enriched ER-, PR-, HER2+ [124,125]	Overexpression of HER2 gene and upregulation of HER2 receptor on cell surface (HER2+). Leads to increased tumour proliferation and aggressiveness and poor prognosis [128,133].	HER2+ cancers are more aggressive. This correlates to an increased ECM/ stroma stiffness profile compared to the Luminal subtypes (1–6 kPa [25]). Furthermore, there was a significant increase in collagen deposition and linearisation compared to Luminal subtypes [25].	Design of 3D scaffolds to mimic the ECM for culture of HER2+ cell lines (*e.g.* AU565 [130])/HER2+ primary cells require a stiffer structure (1–6 kPa [25]) compared to Luminal tumours. Other scaffold alterations could include increased collagen density (*i.e.* scaffold relative density) and production of linearised collagen fibres (*i.e.* anisotropic scaffolds).
Basal/TNBC ER-, PR-, HER2- [124,125]	Predominantly triple-negative tumours (though not exclusively). Aggressive cancers, poor prognosis and difficult to treat [128,134,135].	Basal/TNBC cancers are more aggressive. This correlates to an increased ECM/ stroma stiffness profile compared to the Luminal subtypes (1–6 kPa [25]). Furthermore, there was a significant increase in collagen deposition and linearisation compared to Luminal subtypes [25]. Increased LOX enzyme expression *vs.* non-TNBC cancers [108].	Design of 3D scaffolds to mimic the ECM for culture of Basal/TNBC cell lines (*e.g.* MDA-MB-231 [130])/ Basal/TNBC primary cells require a stiffer structure (1–6 kPa [25]) compared to Luminal tumours. Other scaffold alterations could include increased collagen density (*i.e.* scaffold relative density) and production of linearised collagen fibres (*i.e.* anisotropic scaffolds).

4. Biofabrication techniques for collagen-based breast cancer models

Biologically, collagen-based *in vitro* scaffolds are excellent examples of biocompatible platforms suitable for use in cell culture. In recognition of this fact, there has been a growing interest in the use of collagen-based scaffolds to study a range of cancer types, including breast cancer [4,5,7,27,79,80]. Initial research using collagen-based 3D *in vitro* culture models carried out by Yang et al. in 1979, focused on the use of collagen hydrogels composed of rat tail collagen fibres solubilised in acetic acid [136]. This study demonstrated that dissociated primary mouse breast cancer cells could be successfully maintained over an 8-week period on a 3D *in vitro* collagen hydrogel. The culture model was further validated by transplanting outgrowths from the 3D gels into a murine model where the development of mammary adenocarcinomas, displaying histological similarity to the original tumour from which the primary cells were dissociated from, was observed [136]. Similar success in the growth of mammary tumour cells on collagen hydrogels was reported during a similar time frame (1980–1995) [137–139]. While the success of these early collagen-based 3D *in vitro* models represented an important step forward in breast cancer research, these collagen hydrogels have limited ability to adequately replicate the breast cancer ECM. Recent advances in biofabrication techniques allow greater control over scaffold microarchitecture providing new opportunities for the development of biomimetic 3D breast cancer scaffolds.

In the design of collagen-based scaffolds several criteria must be considered. These include scaffold characteristics such as the architectural, mechanical, degradative, and biological properties. An ideal collagen-based *in vitro* scaffold for breast cancer should demonstrate a high porosity with an interconnected structure and a suitable pore size for cellular and nutrient infiltration and movement throughout the scaffold. The pore size must not be too small, as this can impact cellular migration, and equally the pore size should not be too large as this can reduce cellular attachment due to less scaffold surface area being available [140,141]. Concerning common breast cancer cell lines, MCF7 cells have a reported size (diameter) of ~16–25 μm [142] and MDA-MB-231 cells with a mean cell diameter of 15.81 μm [143] - thus the lower limit of pore sizes for scaffolds culturing these cells must be larger than these values to allow for cell infiltration and migration throughout the scaffold. An optimal pore size or an upper pore size limit has not yet been determined for breast cancer cells. Reported pore sizes with successful culture of breast cancer cells have ranged from 50 to 300 μm [4,5]. Scaffolds must demonstrate mechanical properties similar to native

breast tissue during cancer development [118]. Scaffolds that do not replicate the mechanical properties of native tissue may poorly reflect *in vivo* mechanotransduction [117]. Scaffold stiffness (not limited to breast cancer) has been observed as having a crucial role in driving various cellular pathways and behaviours including proliferation, invasion and differentiation [7,144–146] – thus it is of paramount concern to carefully consider the mechanical properties of the scaffold during the fabrication process. Reported data on the mechanical profile of breast tumour ECM suggests that at a minimum, *in vitro* scaffolds should have stiffness values of $\geq$1 kPa [118,119]. While *in vivo* tumours may have soft cores with stiffness values below 1 kPa [118], the surrounding matrix which is being replicated in these *in vitro* 3D scaffolds requires the increased stiffness in excess of 1 kPa. A benefit of collagen-based *in vitro* scaffolds is the relative ease with which scaffold stiffness can be controlled through crosslinking; therefore *in vitro* scaffolds can be tailored to mimic stiffness values associated with *in vivo* tumour environments. Stiffness profiles of 3D scaffolds could also be tailored to replicate the ECM of specific subtypes of cancers, such as increased scaffold stiffness for culture of aggressive HER2+ and TNBC tumours/cell lines and lower stiffnesses in Luminal tumours/cell lines [25]. Compressive strength of 3D scaffolds is of greater interest during cell culture applications for cancer research in comparison to tensile strength. Culturing cells within 3D scaffolds is known to result in compressive forces between the cells and the surrounding matrix and may potentially result in pore buckling as cells traverse through the scaffold [147,148]. Furthermore, cell/aggregate expansion (representative of tumour expansion *in vivo*) and the resistance provided by the surrounding scaffold generates compressive forces [149,150]. Li et al. recently observed that volumetric compression of 3D organoid cultures induced intracellular crowding which led to increased Wnt/β-Catenin signalling in their intestinal organoid based research [151]. Wnt/β-Catenin signalling plays a crucial role in many cell functions including proliferation, migration, cell fate determination but also has documented roles in cancer [152]. Thus, the Li et al. study emphasises the importance of investigating the interplay between mechanical forces and cell processes, and how 3D models can advance our understanding of the relationship. Freeze-dried collagen-based 3D scaffolds of a 0.5% (weight/volume (*w/v*)) composition have well profiled compressive stiffnesses, exhibiting values of ~0.3–0.5 kPa (standard uniaxial compression, hydrated sample) [153–155]. Crosslinking can increase the compressive moduli values to a range of ~1–2 kPa, crosslinking method and crosslinker concentration dependent [156]. Type 1 Collagen gels of concentrations from 1 mg/mL–7 mg/mL (0.1–0.7% w/v) displayed compressive moduli of ~1.5–8 kPa (standard uniaxial compression, hydrated sample) [157]. Lode et al. reported a mean compressive modulus for their crosslinked 3D printed collagen scaffold of 47.2 kPa (standard uniaxial compression, hydrated sample). Compressive stiffness profiles of collagen structures manufactured through electrospinning are poorly reported. Collagen-based scaffolds should exhibit long-term stability during culture to ensure the maintenance of scaffold integrity over many weeks of *in vitro* investigations (high scaffold mass retention for a minimum of two weeks). Collagen-based scaffolds contain the necessary binding motifs (*e.g.* RGD motifs) to allow for cell adhesion [158], demonstrating biocompatibility and

Table 2
Summary of scaffold fabrication methods.

Fabrication method	Method overview	Scaffold morphology	Advantages	Disadvantages
Hydrogels [79,162,246–249]	Collagen gel solution (typically collagen type 1 and acetic acid) mixed on ice, neutralised (typically NaOH) and then gelated. Variations through parameters; collagen concentration, pH and desired gelation temperature. Gels can be used directly or freeze-dried for storage or analysis purposes.	Dense gel network of string-like fibres. Less defined pore shape and size *vs.* open-cell foam-like structures. Fibre thickness dependent on fabrication parameters.	- Ease of fabrication process. - Matrigel is widely studied and frequently used in cancer research thus many guides for use available. -High level of cell viability reported.	- Less porous than other scaffolds. Can result in poorer cell and nutrient distribution. - More difficult to control architecture thus less reproducibility of exact desired architectures. - Poor mechanical properties prior to crosslinking.
Freeze-Drying [4,140,153,156,250]	Collagen is acid blended (typically acetic acid) at high speed to create homogenous suspension. Suspension then undergoes thermal treatment (controlled or quench) to defined freezing point before returning to ~0 °C for sublimation of ice crystals under vacuum. Dried scaffold ramped to room temperature to complete process.	Highly porous interconnected network; resembles open-cell foam, well defined pore shape and sizes.	- Good control over scaffold architecture through variation in process parameters. Can produce large range of pore size and orientation. - High porosity levels. - Inexpensive. -High level of cell viability reported.	- Can be batch to batch variation in final scaffold architecture due to issues in freezing process. - Poor mechanical properties prior to crosslinking.
Electrospinning [219–225]	Collagen solubilised (typically HFIP or TFE) and added to syringe/injection system. High-voltage electrical field is applied causing solution to be charged and results in polymer fibre erupting from needle tip. Electrical field causes whipping of liquid jet. Solvent evaporates during process leaving a dried fibre network deposited on collection plate (non-woven or aligned).	Dense tightly packed network of fibres (string-like) of nano or micro size. Less defined pore shape and size *vs.* open-cell foam-like structures.	- Production of fibrous network that closely resembles native collagen fibres. - Large range of fibre size/diameter/pattern achievable. -High level of cell viability reported.	- Use of harmful solvents frequent in collagen scaffolds. - Solvents expensive. -Dense fibre networks can reduce level of cell infiltration.
3D Bioprinting [180,236–238]	Collagen ‘ink’ fabricated through mixing of collagen and solvent, commonly PBS or an acid (*e.g.* hydrochloric acid). Ink added to syringe in printing setup and then printed layer-by-layer according to method of printing used and user-controlled parameters. Ink can be printed onto regular surface or into liquid (*e.g.* crosslinking agent to solidify fibres). Printing may be combined with freeze-drying procedure.	One-step printed structures are dense fibrous networks (lower porosity and less defined pore shape and size) while two-step methods combining printing with freeze-drying produces open-cell foam like structure (high porosity and well-defined pore shapes and sizes).	- Control over architecture through use of computer design software. - High porosities achievable. - Use of bioinks for inclusion of live cells into scaffold. -High level of cell viability reported.	-Difficult to print collagen due to viscosity issues and difficultly working at room temperature. - Expensive to scale up. - Often must be combined with a lyophilisation step. - Poor mechanical properties prior to crosslinking.

suitability as a primary scaffold material. The most widely used techniques (Table 2) include hydrogel synthesis, freeze-drying (lyophilisation), electrospinning and 3D bioprinting – all of which produce 3D adherence-based scaffolds or hydrogels. These techniques must achieve the above desired properties for successful application as a breast cancer model.

4.1. Hydrogels

Hydrogels are versatile biomaterial systems with a large range of applications spanning drug delivery, wound repair, tissue engineering and, to a lesser extent, cancer culture. Hydrogels are 3D water-swollen gels consisting of hydrophilic polymers that interact with one another through random interactions (*i.e.* hydrogen bonding) or enforced crosslinks [159,160]. The physical characteristics of gelated hydrogels typically differ from that of scaffolds fabricated using other techniques such as freeze-drying, appearing as a 3D mesh-like network of collagen fibres demonstrating no consistent pore shape or porosity (Fig. 3A). Hydrogel-based scaffolds are generally regarded as less porous structures when compared to their freeze-dried equivalent.

Collagen is the most predominant natural material used for hydrogel synthesis due to its excellent biocompatibility and its abundance within the ECM. Differences in the hydrogel preparation techniques and parameters result in the fabrication of hydrogels exhibiting different properties, which vary in degree of polymerisation, mechanical properties, architecture and biodegradability [159–162]. The architecture of collagen-based hydrogels can be controlled through manipulation of the ionic strength, pH and temperature during gel polymerisation [163,164] – although it is more difficult to control these parameters during hydrogel synthesis in comparison to other scaffold fabrication techniques. Increasing collagen concentration (*i.e.* the gels ionic strength) results in increased fibre density, reduced pore size but has no effect on fibre diameter [164]. Increasing temperature and pH value accelerates polymerisation due to promoting fibre nucleation and electrostatic interactions and results in reduced fibre diameter and smaller pore sizes. Increasing the pH value also increases the mechanical properties of the hydrogel [163,164]. Besides control of the architecture, the mechanical properties of collagen-based hydrogels are also of key interest. Lee et al. (2019) demonstrated collagen hydrogel compressive stiffness values of 1.5–8 kPa [157], demonstrating a wide range of construct stiffness achievable through hydrogel synthesis (through variation of the gels ionic strength in this case). Crosslinking techniques have been utilised to further increase the mechanical properties of the scaffold. Collagen hydrogel crosslinking can be achieved by utilising crosslinking reagents/methods including common chemical methods such as 1-ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC) and glutaraldehyde (GTA) crosslinking [165–167]. Chemical methods can offer a significant increase in stiffness, though such methods may cause cytotoxicity. EDAC is generally well tolerated [168–170] as it is not incorporated into the final scaffold structure thus thorough construct washing prior to use can eliminate cytotoxicity concerns. Nonetheless, at high EDAC concentrations, cytotoxicity and poor cell proliferation has been observed [156,169]. GTA is incorporated into the final scaffold as a result of its crosslinking mechanism and poses a greater cytotoxicity concern [168,171,172] – though again this may be heavily influenced by GTA concentration and can be avoided.

Matrigel is a commercially available hydrogel that comprises of different ECM components including collagen type IV [173]. It has been widely used in cancer research as a versatile platform for the 3D *in vitro* culture of cells [174–176]. While easy to use, batch-to-batch variation can negatively impact research due to differences in hydrogel components and concentrations. Thus, many researchers may prefer to

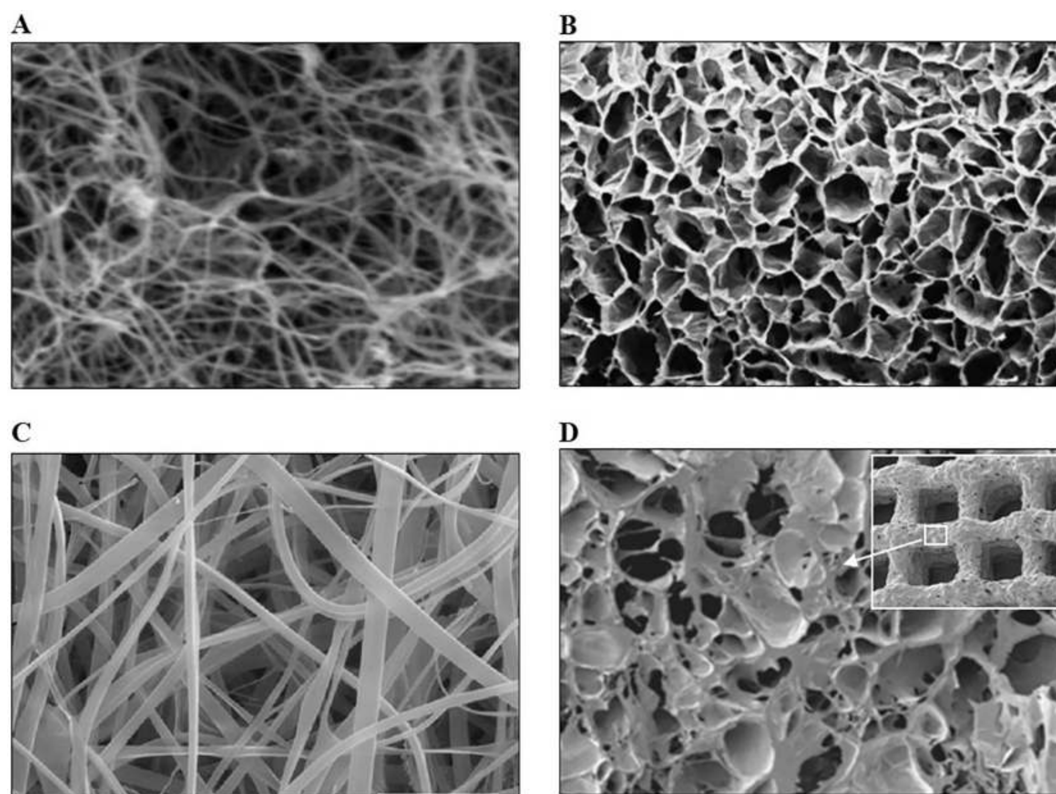

Fig. 3. Comparison of microarchitecture achieved by the four fabrication methods discussed. (A) Collagen hydrogel (adapted from Achilli et al., original licensed under Creative Commons CCBY-NC-SA3.0) [164] (B) Freeze-dried collagen scaffold (adapted from Offeddu et al., original licensed under Creative Commons CCBY-NC-SA3.0) [178] (C) Collagen electrospun scaffold (adapted from Simpson et al., original licensed under Creative Commons CC BY 3.0) [179] (D) 3D printed Collagen scaffold (adapted from Lode et al.) [180].

fabricate their hydrogel systems using defined concentrations. Use of collagen-based hydrogels for breast cancer cell growth is common, including growth of MDA-MB-231 breast cancer cells on type 1 collagen gels [27,79,80] and culture of MCF7 breast cancer cells also on a type 1 collagen gel [177]. Good cell proliferation and viability have been reported for the above examples, demonstrating the use of hydrogels as a viable platform for applications in cancer research.

4.2. Freeze-drying

Freeze-drying or lyophilisation is a dehydration technique, whereby a solution is frozen before undergoing a drying process under vacuum leading to sublimation of formed ice crystals that result in the formation of a dry interconnected, well-defined circular/oval porous microstructure that can be tailored for specific applications (Fig. 3B). This contrasts with electrospun or hydrogel-based scaffolds that have less defined pore shape and consist of long string-like fibre networks. Freeze-drying is currently the most used technique for collagen-based scaffold fabrication. One advantage of fabricating collagen-based scaffolds using the freeze-drying technique is the relative ease that the architecture and mechanical properties can be controlled to more closely replicate the native *in vivo* tumour environment. Controlling the temperature profile of the freeze-drying process can affect the pore structure and size within the scaffold. It has been demonstrated that a lower final freezing temperature results in reduced ice crystal size formation, and therefore a scaffold exhibiting a smaller pore size [181]. The rate of temperature reduction to the final freezing point has also been shown to influence the architecture of the final scaffold. Rapid freezing reportedly results in a scaffold exhibiting a heterogeneous architecture, whereas adopting a slower and controlled freezing rate results in a scaffold demonstrating a homogeneous structure in terms of pore shape and size [182]. The porosity of collagen scaffolds is generally observed to be ~99.5%, which is ideal for tumour cell infiltration and culture [153–155]. Varying the collagen concentration has also been shown to influence scaffold pore size and porosity – increasing collagen concentration from 0.5% to 1% (*w/v*) increased pore size and reduced scaffold porosity [155].

Mould design also plays a key role in determining the final properties of the resultant freeze-dried scaffold. O'Brien et al. report that the use of large rectangular moulds (16.9 × 25.3 cm) results in scaffolds demonstrating poor homogeneity in terms of pore size, shape and alignment when compared to smaller rectangular moulds (12.4 × 12.4 cm) [182]. Further alterations in mould design in terms of material-type and use of secondary mould features, have enabled control of pore alignment, resulting in the development of multidirectional porous collagen-based scaffolds. Isotropic or anisotropic structures have been fabricated by tailoring mould design [4,183]. Isotropic scaffolds have pores of a random arrangement (independent of direction) while anisotropic scaffolds are direction dependant and pores are aligned along one axis. Campbell et al. used a polycarbonate mould with cylindrical wells with pointed copper inserts (PTFE coated). The inserts were thermally insulated from the freeze dryer shelf by a thin 1 mm rubber mat. The features generated a single thermal gradient throughout the collagen slurries resulting in pores aligned in one direction. This ability to tailor the architecture is promising as it could facilitate the fabrication of scaffolds that more closely represent the complexity of component alignment observed in native ECM during breast cancer development, where collagen fibrils frequently linearise [98,115,116].

Freeze-dried collagen-based scaffolds typically have low stiffness properties, *i.e.* ~ 0.3–0.5 kPa [153–155]. Therefore, like with hydrogels, a critical step in the fabrication of freeze-dried collagen-based scaffolds is the inclusion of a crosslinking technique. These include not only chemical methods such EDAC or GTA [156,184,185], but also the use of physical methods such as dehydrothermal treatment (DHT) [156,186] and ultra-violet (UV) [184,187]. Physical methods are well tolerated with no cytotoxic effects. However, chemical methods are considered more robust and offer a greater improvement in scaffold stiffness compared to physical methods [156]. Apart from crosslinking techniques, varying the collagen concentration [155] and *co*-polymerisation of collagen with other materials [153] can also influence the scaffold stiffness. Scaffolds produced *via* freeze-drying have been used more frequently in the culture of breast cancer cells compared to the other techniques discussed in this review [4–7,26,188]. Viable and proliferating cells were observed across all scaffolds with a variety of aspects of breast cancer explored from general proliferation to hypoxia, angiogenesis, invasiveness and response to therapeutic agents. The high porosities, wide range of pore sizes achievable, control over pore alignment and ease of modification of stiffness properties make freeze-dried scaffolds an attractive option for a breast cancer model.

4.3. Electrospinning

The electrospinning process makes use of electrical forces to form fibres from polymer solutions or melts. The resultant electrospun scaffold consists of a network of fibres that offer a large surface area (Fig. 3C), which makes them an attractive platform for cancer cell growth and adhesion [189–192]. The electrospinning technique is fast, efficient, relatively inexpensive and versatile, producing fibres ranging from micrometres [193] to sub-100 nm [194] in diameter. Significant advancements in electrospinning technology and knowledge has allowed for controlled fibre architecture in terms of fibre diameter and alignment. Increasing polymer concentration/viscosity [195–198] can increase the fibre diameter, which also benefits fibre uniformity and reduces the incidence of fibre defects such as beading (low concentration or surface tension issues causing formation of sphere-like defects along fibres) [196,199,200]. Solution conductivity also promotes the production of uniform fibres, with increased conductivity equating to smoother fibres and lower rates of beading [196,199,201]. Reducing the polymer flow rate can also decrease the fibre diameter [196], and increasing the flow rate beyond an optimal value can affect solvent evaporation resulting in highly beaded wet fibres on the collection plate [196,202,203]. The pore size (space between adjacent fibres) within the structure of the electrospun scaffold can be indirectly altered through varying the fibre diameter. Thicker fibres generally increase the pore size and fibres of reduced diameter exhibit the opposite effect due to the higher density of the fibre network within the electrospun scaffold [204]. The inclusion of water-soluble fibres that are sacrificial can increase pore size – these fibres are dispersed homogenously within the main electrospun material and their controlled 'sacrifice' results in the formation of larger pores and a higher porosity [205,206]. High porosity is essential as dense fibrous networks may prevent cell infiltration into the scaffold [205,207,208], thus when generating collagen scaffolds *via* electrospinning, fabrication parameters should be tailored to avoid overly dense tightly packed fibrous networks. Porous fibres have also been developed which further increase surface area due to the fibres themselves containing pores [209,210]. Zhang et al. achieved their porous fibres through leaching of the water soluble constituent of gelatin from PCL/Gelatin composite fibres [209]. Kalra and Tran created porous fibres through high temperature degradation of nafion during carbonization of a polyacrylonitrile/nafion composite scaffold [210]. During conventional electrospinning techniques, collection plates are typically flat surfaces, and due to the 'whipping' action of the charged polymer, the solution collects in a random non-woven pattern. Rotating drum collecting plates have been recently used to generate aligned fibres [211–213]. Other parameters used during the electrospinning process such as surface tension, field voltage, needle tip design and collection plate design can also alter the architecture of the resultant scaffold [214]. Electrospinning techniques have been developed to produce more complex electrospun networks such as multilayer scaffolds, scaffolds with loaded/conjugated compounds (*e.g.* chemotherapeutic conjugation or genetic cargo) [215–218].

Collagen-based scaffolds fabricated using electrospinning have been well documented [219–226]. Typical solvents used during

electrospinning of collagen-based scaffolds include 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) [221,224] and trifluoroethanol (TFE) [219,220] – though nanofibrous collagen-based scaffolds have been spun using a more benign water/salt/alcohol solvent system [223]. Fibre diameter in these collagen-based scaffolds ranged from 100 to 900 nm, with differences achieved through changing the specific electrospinning parameters. Similar to scaffolds fabricated using the freeze-drying technique and hydrogel synthesis, crosslinking agents (*e.g.* EDAC and GTA) can be used to increase the mechanical properties of electrospun scaffolds [220–225]. Excellent cell proliferation and viability have been observed in many collagen-based scaffolds fabricated using electrospinning [221,222,225,227], highlighting their potential as 3D *in vitro* culture scaffold. However, use of electrospun collagen containing scaffolds for breast cancer remains scarce compared to their use in other tissue engineering applications – though where it has occurred, viable breast cancer cell growth and proliferation was demonstrated [207]. This low usage contrasts with synthetic electrospun scaffolds of similar structure which have been used at a greater frequency for breast cancer culture [88–90,228]. Nevertheless, electrospun collagen remains an attractive option due to high level of user control over fibre architecture, rapid production process and the fibrous collagen network achieved has greater resemblance to the fibrous collagenous network of the ECM than the open-cell foam like structures produced by freeze-drying.

4.4. 3D Bioprinting

Since its development in the 1980's, significant advancements in 3D printing technology has resulted in its application in the fields of tissue engineering, regenerative medicine and cancer research [229–231]. 3D printing of biological-based materials has now become a reality and allows for the creation of complex biocompatible 3D structures. Many different techniques are available for the 3D printing of scaffold, including; droplet-based, extrusion-based, inkjet, micro-valve, laser-induced and stereolithography bioprinting [231,232]. Careful control of the design and architecture of the scaffold using available tissue imaging techniques and computer-aided design (CAD) software facilitates the precise and detailed generation of both simple and complex 3D structures. Furthermore, user control over the spatial positioning can facilitate control of the physical, mechanical and signal transduction properties of the resultant 3D printed scaffolds [92,233]. This high level of control may offer a significant advancement in accurately modelling the breast cancer ECM/tumour microenvironment *in vitro*.

Collagen has proven to be a versatile material for bioprinting and has been used in many different 3D printing techniques [190,234–237]. Such collagen-based scaffolds have the classic printed layered lattice appearance, structure and alignment. The struts/fibres of the 3D printed scaffolds normally contain a distribution of micropores on their surface, which facilitates cellular attachment and infiltration, while macropores are located between the layered fibres (Fig. 3D). 3D printing using collagen poses challenges due to the low viscosity of collagen solutions and their inability to solubilise. Therefore, higher viscosity collagen-based solutions have been developed to overcome these issues. Nocera et al. used a highly viscous collagen solution (60 mg/mL collagen in phosphate buffer saline (PBS)) to fabricate fibrillar collagen scaffolds with an interconnected porous structure using 3D printing [238]. The 3D printed scaffold exhibited a porous (≥90% porosity) structure with pores ranging in size from 50 to 500 μm, and cell viability ≥70% [238]. Collagen scaffolds exhibiting highly porous (≥95%) structures with pore sizes ranging from ~10–300 μm have been fabricated using a 3D printing and freeze-drying combination [180,237]. The addition of freeze-drying to the printing process offered scaffold stabilisation and allowed for the development more complex structures. These scaffolds have been shown to offer a suitable environment for the culture and proliferation of keratinocytes, fibroblasts and human mesenchymal stem cells [180,237]. Nerger et al. successfully printed collagen (acid-solubilised bovine type I collagen)–Matrigel composite inks into 3D scaffolds using a microextrusion technique. Scaffolds demonstrated fabrication of spatially controlled aligned collagen fibres. Viable MDA-MB-231 breast cancer cells were cultured when both seeded on top of the scaffolds or when printed within the scaffolds [239].

Another advantage of bioprinting is the potential to incorporate cells within collagen solution to create "bioinks" that allow cells to be seeded within the scaffold during the printing process [232]. This presents a distinct advantage as cells can be dispersed throughout a 3D scaffold during fabrication negating potential risks of poor and slow cell infiltration when manually seeded onto a scaffold surface. Collagen-based bioinks containing cells such as fibroblasts [240,241], MSC cells [242], osteoblasts [234,243] and hepatocytes [241] have been well documented and have demonstrated good/excellent cell viability and proliferation. As with other collagen-based *in vitro* scaffolds, different crosslinking options are available to increase the mechanical properties of 3D printed collagen-based scaffolds including EDAC [180,237], genipin [234] and tannic acid [244,245]. While 3D printing has been minimally used in the development of collagen-based 3D models for breast cancer [239], it nonetheless holds significant potential to produce models that more accurately model the breast cancer tumour microenvironment.

5. Application of 3D collagen-based scaffolds in *in vitro* breast cancer research

Collagen-based platforms have been utilised in a range of *in vitro* breast cancer models aimed at advancing the current understanding of breast cancer development and progression. A selection of these studies are discussed herein (Table 3). Initial validation and confirmation of platform biocompatibility is common throughout these studies, but recently studies have focused on complex investigations into various breast cancer phenomena. Some areas of study include alterations in cell morphology and growth kinetics, assessment of therapeutic response, genetic and metabolic analysis, metastasis and the interplay between matrix stiffness and a variety of cell properties.

Several studies have focused on the validation of collagen-based 3D *in vitro* culture scaffolds with general confirmation of breast cancer cell growth and assessment of cell behaviour within the 3D platforms. The MCF7 breast cancer cell line was successfully grown in a 3D collagen scaffold fabricated using a freeze-drying technique. Cells in this 3D *in vitro* scaffold displayed similar proliferation rates to 2D cultures during the initial days, though culture life was longer in the 3D models as 2D cultures reached confluency more quickly and began undergoing apoptosis. Cells in 3D also displayed a more rounded morphology than those in monolayer. Furthermore, the MCF7 cells demonstrated increased malignancy and an invasive phenotype compared to when grown in 2D [6]. There was an increased expression of proangiogenic growth factors and MMPs within the 3D scaffolds [6] – expression signatures such as these are common in clinical malignant cases [111,113]. The increased malignant-like behaviour of the cells grown in 3D was confirmed through xenograft development – tumours derived from 3D cultured cells were significantly larger and displayed increased tumorigenicity compared to 2D culture-derived xenografts. A further interesting observation was the display of stem cell-like properties and markers within the MCF7 population in 3D cultures compared to their 2D counterparts [6]. Electrospun collagen scaffolds have rarely been used for breast cancer research, despite frequent use elsewhere in tissue engineering. In one study, MDA-MB-231 breast cancer cells were cultured on an electrospun scaffold composed of a 1:1 ratio of collagen type 1 and PCL with varied weight/volumes of 5–15%. Fibre diameters ranged from ~400 nm to 2.25 μm, concentration dependent. Breast cancer cells showed sustained viability and successful proliferation on the electrospun scaffolds. However the dense fibre network of the scaffolds significantly impacted cell infiltration, resulting in few cells infiltrating to the centre of the scaffold [207]. To overcome this,

Table 3
3D collagen scaffolds as breast cancer models.

Scaffold type	Methodology overview	Properties (if reported)	Main findings	Rerefence
Collagen hydrogel	Rat-tail derived collagen, solubilised in acetic acid; gelated at room temperature, culture of primary mammary cells from murine model.	Not reported	Maintenance of primary mammary cells over 8-week period. Histological similarity to original mammary tumours.	Yang et al. (1979) [136]
	Collagen type 1 (rat tail) solubilised in 10× DMEM, 1 N NaOH and distilled water to a final concentration of 8 mg/mL. Gelation at 37 °C. MDA-MB-231 breast cancer cells cultured on hydrogels of 1.5 or 3 mm thickness.	Not reported	Hypoxic conditions were observed *in vitro* that showed similarity to *in vivo* breast tumours. Oxygen/nutrient gradients were observed along with necrotic regions. There was a significant upregulation in HIF-1α and VEGF-A in the 3D gel compared to 2D cultures.	Szot et al. (2011) [79]
	Type 1 collagen (rat tail) mixed with DMEM, acetic acid and NaOH for final gel concentration of 0.3 mg/mL. Gelation at 37 °C. Core biopsies of ER+ or ER low/- tumours cultured within gels.	Not reported	Successful *ex vivo* culture of patient tumour samples was demonstrated within the type 1 collagen gels. The expected response upon tamoxifen exposure was also demonstrated, with ER+ tumours undergoing tumour regression and apoptosis where ER low/- tumours showed little to no response.	Leeper et al. (2012) [262]
	Two collagen gels (rat tail type 1 collagen); a low stiffness gel with a concentration of 1.2 mg/mL gel and the other a high stiffness at 2.8 mg/mL. Gelation at 37 °C. T47D and MCF7 breast cancer cells cultured.	Not reported	The study was an investigation on the interplay between matrix stiffness and prolactin signalling. In the high stiffness gel; ERK1/2 signalling was favoured, increased expression of MMP-2 in both cell lines, linearization of collagen fibres within the gels cultured with T47D cells and a general increase in invasive and disorganised behaviour by T47D cells. Such results were not seen in lower stiffness gels, highlighting the tumourigenic properties of prolactin in stiffer environments.	Barcus et al. (2013) [177]
	Type 1 rat-tail collagen in HEPES/PBS and diluted with DMEM; gel concentration of 1–4 mg/mL. Group of gels mechanically strained (prestrained) to linearise collagen fibres. Gelation at 37 °C. MDA-MB-231 breast cancer cells cultured on gels to assess migration.	Tensile strength of 2 mg/mL collagen gels; prestrained - ~15–25 kPa. Nonstrained - ~3–5 kPa.	Aligned collagen fibres resulted in an increase in net migration distance travelled by MDA-MB-231 cells. The aligned architecture did not affect migration speed of cells. Stiffness did not influence migration compared to the aligned architecture.	Riching et al. (2015) [80]
	Rat-tail tendon type I collagen solubilised in 0.6% acetic acid. Mechanically compressed to form dense hydrogel of 6.074 ± 1.82 wt% gel. Gelation at 37 °C. Co-culture of MDA-MB231 breast cancer cells & MC3T3-E1 pre-osteoblastic (bone cell) cell line.	Not reported	Investigation of the effect of osteolytic breast cancer cells on osteoblast activity/differentiation. Co-culture of the MDA-MB-231/MC3T3-E1 cells or exposure of MC3T3-E1 (bone cell line) cells to MDA-MB-231 conditioned media resulted in impairment of MC3T3-E1 differentiation to osteoblasts and subsequent reduction of osteoblast-mediated mineralisation.	James-Bhasin et al. (2018) [27]
Collagen Scaffold; Freeze-Dried	Collagen membranes solubilised in 0.5 M acetic acid. Lyophilised and cut to 0.1 × 0.5 × 0.5 cm pellets. EDAC/NHS crosslinked. Culture of MCF7 cells.	Not reported	Enhancement of MCF7 stem cell markers. Increased MMP transcription and increased pro-angiogenic factors. Overall better simulation of *in vivo* breast cancer cell morphology & markers in comparison to 2D culture.	Chen et al. (2012) [6]
	05% (*w/v*) collagen (type I, bovine tendon) & 0.05% (w/v) chondroitin-6-sulfate (isolated from shark cartilage) solubilised in 0.05 M acetic acid. Lyophilised and then DHT crosslinked at 105 °C. Mammary adenocarcinoma 4 T1 cells cultured.	Not reported	4 T1 cells were capable of osteomimicry and showed enhanced mineralisation properties, suggesting mammary cancer cells can adapt to the bone environment – a frequent site of breast cancer metastases.	Cox et al. (2012) [26]
	A 3D biomatrix (GELfoam) was used; 5 mm in diameter and 3 mm in thickness. Breast cancer cell lines SUM159, SUM149, MDA-MB-231, MDA-MB-435, BT474, MCF7, T47D and ZR75–1 used. Primary human bone marrow cells used alongside immortalised bone marrow cell lines.	Not Reported	This study focused on the relationship between breast cancer cells and bone cells. Breast cancer cell lines (8 cell lines) and bone marrow cells were co-cultured. The development of supportive (allowed for proliferation) and inhibitory niches (induced dormancy) to breast cancer cell proliferation by varying the co-cultures on the scaffolds was observed.	Marlow et al. (2013) [254]
	1% (w/v) Collagen (bovine achilles tendon) solubilised in 0.05 M acetic acid. Tailored freeze-drying procedures to produce both isotropic and anisotropic scaffolds. EDAC/NHS crosslinked. Culture of MCF7, MDA-MB-468 and MDA-MB-231 breast cancer cells.	Pore size 100 μm	The anisotropic scaffold significantly increased the migration and invasion capacity of invasive MDA-MB-231 cells compared to isotropic scaffolds. Non-invasive MCF7 cells migrated a significantly smaller distance through either scaffold. MDA-MB-468 cells that underwent epithelial-to-mesenchymal transition (EMT) showed enhanced migration in anisotropic scaffolds.	Campbell et al. (2017) [4]
	1% (w/v) bovine collagen (type I) in acetate buffer suspension. Scaffolds crosslinked with 1,4-butanediol diglycidyl ether solution then freeze-dried. MDA-MB-231 and MCF7 breast cancer cells used.	Porosity of 87.8% and pores within a range of 150 to 300 μm.	Both cell lines formed tissue-like 3D features and maintained expected morphology. MDA-MB-231 cells caused a significantly stiffer scaffold environment with increased collagen content and increase LOX expression. Treatment with LOX inhibitor β-aminopropionitrile resulted in impaired MDA-MB-231 cell influence on scaffold stiffness.	Liverani et al. (2017) [7]
		Pore size 100 μm		

(*continued on next page*)

Table 3 (*continued*)

Scaffold type	Methodology overview	Properties (if reported)	Main findings	Rerefence
	1% (w/v) collagen (bovine achilles tendon) solubilised in 0.05 M acetic acid. Tailored freeze-drying to produce anisotropic scaffolds. EDAC/NHS crosslinked. Culture of xenograft tumour fragments and co-culture of fragments with 3 T3-L1 preadipocyte cell line.		Xenograft breast tumour samples were successfully cultured within collagen scaffolds highlighting *ex vivo* application promise. Adipocytes found to increase migration of tumour cells within the scaffolds at Day 10. Canertinib treatment (chemotherapeutic) resulted in significant impairment of tumour cells to migrate into the scaffold, both distance wise and number wise.	Hume et al. (2018) [188]
	1% (w/v) bovine collagen (type I) in acetate buffer suspension. Scaffolds crosslinked with 1,4-butanediol diglycidyl ether solution then freeze-dried. MDA-MB-231 and MCF7 breast cancer cells used.	Porosity of 84.8% ± 6.3.	Cells cultured within the collagen scaffolds drove formation of hypoxic microenvironment with subsequent increase in hypoxia-driven cell behaviours that resembled *in vivo* breast tumours. Cells acquired more aggressive phenotype than 2D monolayer with increased LOX expression, enhanced migratory ability, induction of glycolysis and increased secretion of pro-angiogenic factors.	Liverani et al. (2019) [5]
Collagen/PCL Scaffold; Electrospinning	1:1 weight ratio of collagen type 1 and PCL solubilised in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at varied weight/volume concentrations of 5–15%. Electrospun onto spinning mandrel. MDA-MB-231 cells cultured on scaffolds.	Fibre diameters ranged from ~400 nm (5%) to 2.250 μm (15%), concentration dependent	This study assessed cell behaviour on fabricated collagen/PCL electrospun fibres in terms of growth, proliferation, adhesion and infiltration. MDA-MB-231 cells showed high levels of viability and successful proliferation. However, infiltration was hampered by density of scaffold fibres.	Szot et al. (2011) [207]

techniques to improve infiltration and increase pore size of the scaffold would be required, such as altering solution concentration [197] or use of sacrificial fibres [205]. While this study demonstrated the potential of electrospun scaffolds to support breast cancer cell growth, to date electrospun scaffold-based 3D scaffolds have not been used in to explore breast cancer cell behaviour or in drug screening studies.

5.1. Breast cancer and bone metastases

Bone is the most common site of metastasis in breast cancer for all subtypes – though progression to bone metastasis from initial breast cancer diagnosis is faster in the more aggressive HER2+ and TNBC subtypes [251,252]. Once breast cancer has spread to the bone, it is considered incurable [253] - thus greater attention is urgently needed to develop new approaches to increase survival and eventually to discover potential cures. 3D *in vitro* scaffolds provide a useful tool for the investigation of metastasis. James-Bhasin et al. (2018) developed a 3D *in vitro* co-culture consisting of osteolytic breast cancer MDA-MB-231 cells and pre-osteoblast MC3T3-E1 cells within a dense collagen hydrogel (rat tail collagen type 1 solubilised in acetic acid) in order to investigate the interaction between triple negative breast cancer cells and osteoblasts, [27]. They reported that the co-culture of the MDA-MB-231 and MC3T3-E1 cells resulted in an impairment of the differentiation of MC3T3-E1 cells to osteoblasts and subsequently a reduction of osteoblast-mediated mineralisation [27]. A similar effect was observed on exposure of MC3T3-E1 cells to MDA-MB-231 conditioned media. The suppression of osteoblast activity by breast cancer cells in this study is of interest as it may be a potential target for the therapeutic reduction of bone density loss in metastatic breast cancer. Murine mammary adenocarcinoma 4 T1 cells have also been successfully grown on a 3D collagen-glycosaminoglycan freeze-dried scaffold in an investigation to assess the adaptability of breast cancer cells to the bone microenvironment, which may explain why bone is a preferential site of metastasis for breast cancers [26]. The cells were capable of osteomimicry and showed enhanced mineralisation properties, further highlighting the potential of 3D *in vitro* collagen-based scaffolds in the study of breast cancer cells and their high rate of bone metastases [26]. A further study focusing on the interplay between breast cancer cells and bone cells used a 3D collagen scaffold to increase understanding in breast cancer cell dormancy and eventual metastasis [254]. A range of breast cancer cell lines (8 cell lines total - SUM159, SUM149, MDA-MB-231, MDA-MB-435, BT474, MCF7, T47D and ZR75–1 cells) and bone marrow cells were successfully grown in co-culture on the scaffolds. Significant findings from this study were the development of supportive and inhibitory niches to breast cancer cell proliferation by varying the co-cultures on the scaffolds. Primary bone marrow stromal cells supported breast cancer cell proliferation while bone marrow cell lines (including osteoblast, mesenchymal, and endothelial cell lines) suppressed proliferation of breast cancer cells and induced dormancy. The ability to model dormancy *in vitro* may lead to eventual biomarker discovery concerning dormant disseminated cells that may cause metastasis upon proliferation at distant sites [254] – discovering these biomarkers and assessing patients for them may offer a new frontier in predicting metastasis risk.

5.2. Collagen alignment and cell Invasion

A signature of breast cancer development and increased invasiveness is the linearisation of collagen fibres, creating 'highways' for cells to migrate along away from the primary tumour [23,115]. This linearisation is seen increasingly in aggressive cancer subtypes including HER2+ and TNBC tumours [25] thus is an important point of investigation. In an attempt to investigate the development of a new migration/invasive potential assay, Campbell et al. [4] developed 3D collagen-based scaffolds demonstrating both direction aligned (anisotropic) and non-directional random (isotropic) pore architectures [4]. In this study a freeze-dried collagen scaffold (bovine achilles tendon derived collagen type 1 solubilised in acetic acid) was fabricated with axially aligned pores as it was hypothesised that such scaffolds could better mimic the *in vivo* linearised collagen fibres. Pores within this scaffold were ~100 μm in size. Data from this study showed that the anisotropic scaffold significantly increased the migration and invasion capacity of invasive breast cell line MDA-MB-231 when compared to the isotropic scaffold equivalent. High numbers of MDA-MB-231 cells migrated through the full depth of the collagen-based scaffold, whereas non-invasive MCF7 cells travelled a significantly lower migration distance through the scaffold [4]. The above anisotropic scaffold was further applied for migratory analysis in a study investigating xenograft tumour invasiveness and response to chemotherapeutics. The study increased replication of the breast ECM through culture of adipocytes within the collagen scaffolds prior to seeding of tumour fragments [188]. Adipocytes are a prevalent cell group within mammary tissues and are reported to play a role in breast cancer invasion [255,256]. Following 10 days of tumour fragment culture, adipocytes were found to increase migration of tumour cells within the scaffolds. Treatment of cultures with chemotherapeutic canertinib (tyrosine kinase inhibitor) resulted in significant reduction of tumour cell migration into the scaffold, both distance wise

and number wise [188]. Such results further emphasise the potential of collagen *in vitro* models for drug development and discovery within cancer research. Furthermore, the successful culture of xenograft tumour fragments showed the potential in application of these models for *ex vivo* culture of patient tumour fragments. This could facilitate advancements of personalised treatment strategies through investigation of tumour properties and response to therapies on a patient-by-patient basis. A further 3D collagen model highlighted the key role aligned collagen fibres play in cancer cell migration. Aligned collagen hydrogels resulted in an increased net migration distance through the constructs by MDA-MB-231 cells when compared to randomly organised collagen gels. The study also reported that collagen fibre alignment was the dominant factor affecting the migration distance, leading to a greater increase in migration distance than matrix stiffness [80].

5.3. Scaffold stiffness & breast cancer interplay

Stiffness of the ECM is a key prognostic feature of breast cancers and collagen plays a key role in this matrix stiffening [24,96,97,104]. While it is generally accepted that stiffness plays a role in progression and spread of breast cancer, the exact mechanisms of the influence of stiffness remain poorly understood. Barcus et al. (2014) sought to understand the role of matrix stiffness on prolactin signalling in breast cancer cells [177]. Prolactin levels are associated with breast cancer progression [257–259] and as a result prolactin has attracted much interest in breast cancer research, though its exact actions and role in the disease is not fully understood. In this study, two collagen gels (rat tail type 1 collagen) of different stiffness profiles, one a low stiffness 1.2 mg/mL gel and the other a high stiffness 2.8 mg/mL gel, were fabricated. Both T47D and MCF7 breast cancer cell lines were cultured. The stiffer collagen gels led to prolactin induced stimulation of ERK1/2 signalling pathways in both cell lines (potential protumourigenic role), increased expression of MMP-2 in both cell lines (key factor in ECM degradation and spread of breast cancer [111–114]), linearisation of collagen fibres within the gels cultured with T47D cells (assists metastasis and is a signature of poor breast cancer prognosis [98,115]) and a general increase in invasive and disorganised behaviour by T47D cells. Similar effects of prolactin were not observed in the lower stiffness collagen gel, demonstrating the key role of stiffness in directing cell signalling/activity and its association with favourable conditions for cancer spread and progression [177]. Liverani et al. further investigated the mechanobiology of breast cancers through culture of two breast cancer cell lines, MCF7 (non-invasive) and MDA-MB-231 (invasive), on collagen scaffolds. Scaffolds consisted of a 1% (*w/v*) bovine collagen (type I) suspension, prepared with an acetate buffer. The scaffolds were crosslinked with an 1,4-butanediol diglycidyl ether solution before undergoing a freeze-drying procedure, producing an interconnected porous structure (porosity of 87.8% and pores within a range of 150 to 300 μm). Upon culture, both cell lines formed tissue-like 3D features and maintained expected morphology. The more aggressive MDA-MB-231 cells caused a significantly stiffer scaffold environment with increased collagen content and increased LOX expression – such properties resemble *in vivo* tumours generated from MDA-MB-231 cells. Upon treatment of MDA-MB-231 cells with LOX inhibitor β-aminopropionitrile, the cells ability to increase scaffolds stiffness was impaired [7]. Due to the correlation between increased ECM stiffness and poor patient prognosis [19,117], *in vitro* models such as the above can serve as a useful investigational tool for cell-ECM interactions and their influence on patient tumour behaviour.

5.4. Hypoxic environment generation and investigation

Hypoxia is a key factor in breast cancer progression and metastasis risk [260,261], thus it is important to investigate the development of hypoxia in a relevant 3D microenvironment *in vitro* and its influence on key genetic and cellular behaviours. A collagen hydrogel (type 1 extracted from rat tail) demonstrated generation of hypoxic conditions *in vitro* that showed similarity to *in vivo* breast tumours. MDA-MB-231 cells were cultured on 3 mm thick collagen gels and generated spheroid like clusters within the gels with oxygen/nutrient gradients present and the development of necrotic regions was observed. Compared to 2D culture, there was a significant upregulation in hypoxia-inducible factor (HIF)-1α (hypoxia marker) and vascular endothelial growth factor (VEGF)-A (angiogenesis marker) [79]. Liverani et al. also demonstrated the development of a hypoxia model with high mimicry to *in vivo* tumours using a freeze dried collagen scaffold (type 1 collagen solubilised in acetic acid, crosslinked with 1, 4-butanediol diglycidyl eter, average porosity of 84.8%) [5]. Through modelling the low oxygen environment typical of primary tumours, they were able to successfully investigate a range of hypoxia-driven cell behaviours including proliferation, aggressiveness, senescence and metabolic activity. Culture of MCF7 and MDA-MB-231 breast cancer cells within the scaffolds resulted in generation of a tissue-like environment and ECM secretions by the cancer cells resulting in a scaffold microenvironment that closely corresponded with an *in vivo* mouse comparator. Both cell lines showed development of a hypoxic niche with high HIF-1α expression, upregulation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and successful pimonidazole staining (stains for poorly oxygenated cells). Furthermore, LOX enzyme expression and VEGF secretion was significantly higher in the 3D scaffolds when compared to monolayer 2D culture [5]. Models such as the above hydrogel [79] and scaffold [5] platforms that can accurately replicate *in vivo* tumour hypoxic environments provide a powerful *in vitro* tool for investigation into breast cancer progression and for therapeutic targeting of hypoxia associated cell invasiveness.

5.5. Scaffold use in drug screening

A key application of 3D scaffolds is in drug screening and discovery. 2D cultures are known for altered or inflated response to therapeutics, with poor translation of drug efficacies seen in 2D cultures when they progress to animal models [39–43]. Use of collagen scaffolds for drug screening or discovery for breast cancer is at a low level at present, though the area offers huge potential and is a key factor in 3D *in vitro* platforms bridging the gap between 2D culture and animal xenografts. In one study, patient tumour fragments were cultured on a collagen gels (type 1, rat tail, 0.3 mg/mL) for *ex vivo* culture validation and tamoxifen (Estrogen Receptor (ER) + treatment) assessment [262]. Patient tumours maintained high levels of cell viability in culture and ER+ tumours showed a measured response to tamoxifen treatment *versus* untreated counterparts (ER+ tumour fragment with no tamoxifen exposure). Low ER or ER- tumours showed no significant reduction in tumour volumes with tamoxifen treatment compared to untreated counterparts (ER low/- tumour fragment with no tamoxifen exposure). These responses/lack of responses correspond with expected tamoxifen action in human patients, demonstrating the useful application of collagen platforms for drug screening and also as an *ex vivo* platform [262]. MCF7 spheroids embedded and unembedded in collagen gels treated with anticancer compound doxorubicin displayed increased resistance to drug-mediated cytotoxicity in comparison to 2D monolayer of MCF7 cells [263]. Reduced susceptibly to drug compounds in 3D platforms is a key advantage of their use compared to 2D monolayer and allows for increased *in vitro* correlation to typical animal model/human response profiles.

6. Concluding remarks and future perspectives

Emergence of the use of 3D *in vitro* models over the last two decades for different applications has seen a promising shift away from the heavy reliance on 2D *in vitro* culture. While collagen scaffolds have been widely used in 3D culture in tissue engineering and regenerative medicine research, their potential in the field of oncology and research is still in the nascent stage. A range of fabrication techniques (*e.g.* hydrogel

synthesis, freeze-drying, electrospinning and 3D printing) are currently used to produce collagen-based scaffolds. Each technique confers their own advantages and disadvantages (Table 2) and depending on the end application and the desired architecture/final properties the most suitable method varies. Collagen-based scaffolds fabricated using freeze-drying, electrospinning and 3D bioprinting offer many advantages when considering replicating breast cancer tissue, *e.g.* demonstration of a highly interconnected porous structure, ease of fabrication and ability to tailor the physical, mechanical and biological properties. Similar platforms produced by hydrogel synthesis demonstrate structures of lower interconnectivity, porosity and poorer reproducibility making them a less attractive option. While the fibrous network of electrospun scaffolds is attractive as it captures the fibrous collagen network seen in the breast cancer microenvironment, the poor cellular infiltration in these scaffolds is a significant disadvantage of their application. While collagen-based scaffolds fabricated using freeze-drying or hydrogel synthesis have been most widely used in breast cancer research to date, recent developments in 3D bioprinting and additive manufacturing techniques offer enormous potential in terms of their ability to produce scaffolds with precisely controlled microarchitectures. Thus, the use of 3D printing techniques in the development of 3D scaffolds for breast cancer research is likely to increase significantly in the coming years. To increase the appeal of 3D models for cancer research, increased replication of the human ECM and tumour microenvironment is essential. Inclusion of further ECM components including fibronectin, laminins, other collagen types, hyaluronan and more [24] in collagen scaffolds is feasible with the discussed fabrication techniques and should be considered moving forward. Through improving the replication of ECM in these *in vitro* scaffolds, combining them with other 3D culture techniques such as spheroid or organoid culture, may offer new leading research tools in breast cancer research with high mimicry of *in vivo* tumours with the provision of complex tumour-stroma interactions. Furthermore, inclusion of immune function in these 3D models could offer a significant advancement in *in vitro* modelling of breast cancer, allowing for key interactions between cancer cells and immune cells and more advanced drug screening involving drugs that utilise immune function.

Successful culture of cancer cells has been achieved using different collagen-based scaffolds (Table 3). High cell viability, stable proliferation over days/weeks in culture and formation of spheroids within the 3D *in vitro* scaffolds highlight the promise of collagen-based scaffolds in understanding cancer development and progression. 3D collagen-based *in vitro* models have also shown their applicability as *ex vivo* culture scaffolds [136,188]. Such a use moving forward would enable the rapid *ex vivo* culture of patient tumour samples, thus revolutionising personalised medicine strategies by enabling best fit treatments to be devised on a patient-by-patient basis. In the future, continued research needs to be directed towards the application of these 3D collagen-based *in vitro* models in the exploration of breast cancer phenomena including: the role the ECM plays in cancer progression and spread in terms of stiffness and architectural properties, growth/proliferation kinetics and angiogenic/metastatic potential. Furthermore, their application and use in drug discovery, development and screening must be further explored and this could potentially have a positive impact for breast cancer treatment and ultimately global health.

CRediT authorship contribution statement

John Redmond: Conceptualization, Writing- Original draft preparation, Writing- Reviewing and Editing. **Helen McCarthy:** Writing-Reviewing and Editing. **Paul Buchanan:** Supervision, Writing-Reviewing and Editing. **Tanya Levingstone:** Supervision, Writing-Reviewing and Editing. **Nicholas Dunne:** Supervision, Writing-Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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