Collagen as extracellular matrix (ECM) for three-dimensional (3D) cell culture models: A mini-review

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ABSTRACT In vitro cell culture models are crucial tools in drug discovery for understanding pathophysiology and cellular physiology. Traditionally, two-dimensional (2D) cultures have significantly advanced cell-based research. However, their translational potential is limited by their inability to replicate the natural cellular environment. The emergence of three-dimensional (3D) culture models offers more physiological relevance platforms to mimic a natural tissue architecture. Scaffold-based techniques that utilize extracellular matrix (ECM) components have become increasingly prominent, with collagen emerging as a leading candidate due to its structural and biological properties. Collagen, the key player of structural protein in connective tissue, may act as biomimetic scaffolds that facilitate cell aggregation, proliferation, and migration. This mini-review explores the unique features of collagen as a hydrogel scaffold, including its biocompatibility, natural abundance, and the capacity to replicate tissue-specific architectures.

KEYWORDS: 2D and 3D cultures; In vitro; Scaffolds; Collagen; ECM
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Review Article

INTRODUCTION

In vitro mammalian cell cultures provide defined platforms to understand cell physiology and pathophysiology outside the organism (Duval *et al.*, 2017). Two-dimensional (2D) culture model has been typically used in cell-based studies. However, cells display native behaviour in 3D environments (Cacciamali *et al.*, 2022). Cell morphology, physiology, metabolism, and stimuli response are crucial aspects of cell growth (Su *et al.*, 2024). Traditionally, the 2D culture model has been widely utilized in cell-based studies, particularly in drug discovery, significantly advancing our understanding of drug mechanisms of action. However, the 2D models have critical limitations where cells are grown on a culture flask that is made up of a stiff platform, leading to unnatural growth kinetics and cell attachments (Bédard *et al.*, 2020). As a result, the natural microenvironment of the particular cells is not fully represented, which could render misleading data. To address these challenges, focus has been shifted to 3D cell culture system as the technique is designed to accurately mimic tissue-specific microarchitectures and cellular behaviour (Ballav *et al.*, 2021). This system utilizes both scaffold-based or scaffold-free-based techniques. Scaffold-based culture is a technique that provides mechanical support to the extracellular matrix (ECM) in which cells may aggregate, proliferate, and migrate (Ravi *et al.*, 2015). Collagen, one of the key components of ECM, has emerged as a promising scaffold material for creating a reliable 3D microenvironment model due to its structural and biological features. Thus, this mini-review aims to explore the use of collagen as a potential scaffold for ECM to mimic a reliable 3D microenvironment model.

TRANSACTIONS ON SCIENCE AND TECHNOLOGY

3D CELL CULTURE MODEL

The establishment of the 3D culture involves scaffold-based and scaffold-free-based techniques. Scaffold-based culture is a technique that provides mechanical support to the ECM in which cells may aggregate, proliferate, and migrate (Ravi *et al.*, 2015). The primary natural materials utilised in ECM include collagen, fibrin, gelatine, elastin, chitosan, chitin, and hyaluronic acid (Ravi *et al.*, 2015). The conventional approaches used in scaffold-based cultures are hydrogels and solid-state scaffolds. On the contrary, a scaffold-free system is a technique that facilitates the development of multicellular aggregation (Khoshnood *et al.*, 2021). Both techniques could be applied to two distinct cell features, such as spheroid and organoid formations. The 3D spheroid model involves cell aggregation from either single or multicellular types (Gunti *et al.*, 2021). They mainly originate from the immortalised cell lines but can also be established using primary cells (Gunti *et al.*, 2021).

In contrast, organoid culture represents a model that is typically developed from single adult stem cells or embryonic stem cells (Huang *et al.*, 2021; Clevers, 2016). Moreover, it is referred to as a miniature version of organs that display definite microanatomy that is similar to an in vivo model. Table 1 highlights the differences between 2D and 3D cell culture models; meanwhile, Figure 1 depicts the characteristics of 3D cell culture features in an ECM environment compared to the monolayer method.

Table 1. Comparison between traditional 2D and 3D cell culture models.

Cell features	2D monolayer	3D spheroid/organoid
Time	Minutes to hours	Consume hours to days
In vivo imitation	Do not mimic the natural environment	Mimics in vivo environment
Cell interactions	Lack of cell interactions	Proper interactions of cell-cell and formation of environment niches
Characteristics of cells	Changed morphology and lack of phenotypic variety	Preserved morphology and has diverse phenotype and polarity
Metabolism uptake	Unlimited access to oxygen, nutrients, metabolites and signalling molecules	Variables access to oxygen, nutrients, metabolites, and mimics *in vivo* environment
Molecular mechanism	Changes in gene expression and biochemistry of cells	Gene expression, mRNA splicing, topology and biochemistry as in vivo
Cost	Cheap	Expensive

Adapted from Kapałczyńska *et al*. (2018).

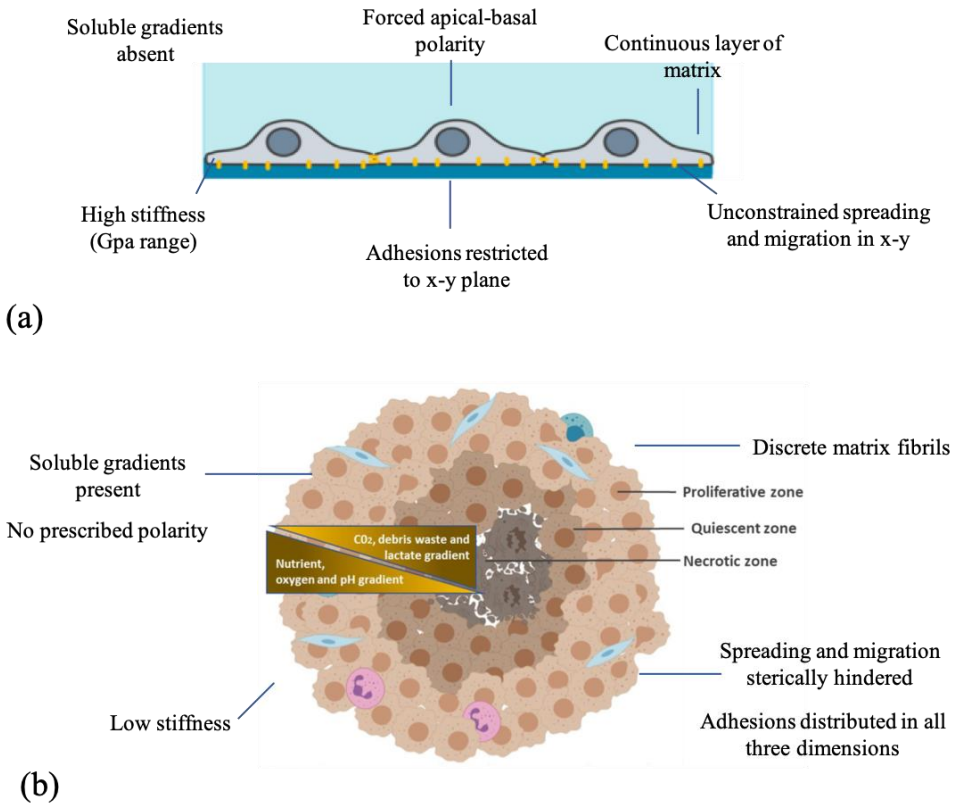

Figure 1. A comparison of cellular growth in 2D (a) and 3D (b) cell cultures. A typical ECM-like collagen facilitates the formation of spheroid cells similarly to the in vivo model. However, the 2D monolayer culture limits the cell distribution and growth, which could affect the drug's effectiveness, thus providing misleading data. Adapted from Biju *et al.* (2023).

SCAFFOLD-BASED HYDROGEL

Scaffold-based hydrogels are distinctive as they permit soluble factors like cytokines and growth factors through a network of tissue-like gels. Hydrogel is a water-based polymeric material that consists of strong crosslinked junctions that are formed through hydrogen bonding (Huang *et al.*, 2017). The polymer in hydrogels consists of hydrophilic networks that facilitate the absorption and retention of large quantities of water (Daniele *et al.*, 2014). It is one of the most notable scaffolds as it naturally mimics the ECM in our body. The soft-rubbery texture, low surface tension, and abundant water content resemble a suitable alternative to natural tissues (Bonferoni *et al.*, 2021).

Hydrogels, which consist of natural, synthetic, and hybrid materials, provide a wide range of chemical and mechanical functions (Joseph *et al.*, 2018). They provide adhesive properties, promote cell viability, and enable cell proliferation and differentiation (Joseph *et al.*, 2018). A natural polymer such as collagen is the most extensively used for hydrogel preparation as it is ubiquitous in tissues like ligament, cartilage, bone, skin, and tendon (Bonferoni *et al.*, 2021). Synthetic polymers serve biological properties similar to natural hydrogels. Only then they have improved gel consistency which is an ideal choice of material in 3D scaffolds (Joseph *et al.*, 2018). The synthetic materials used for hydrogel formulation consist of chitosan derivatives such as polyacrylic acid, polyethylene glycol (PEG), polyglycolic acid (PGA), polyvinyl alcohol, and poly(2-hydroxy ethyl methacrylate) (pHEMA) (Gibbs *et al.*, 2016). These materials are frequently used for their durability, excellent gel strength, and water absorption capacity (Hoffman, 2012). Besides, synthetic materials have a well-defined architecture for gel stiffness and porosity (Zhang & Khademhosseini, 2017).

SCAFFOLD REQUIREMENTS FOR ECM APPLICATION

There is a vast number of scaffolds produced through plethora fabrication techniques in efforts to reconstruct tissue regeneration in the body (O'brien, 2011). However, several essential features must be considered to ensure scaffold compatibility in tissue engineering, irrespective of tissue type. The very first criterion is that they must be biocompatible i.e. the cells must have adherent properties, the ability to migrate onto the surface of the scaffold, and initiate proliferation (O'brien, 2011; Zidan *et al.*, 2018). For organ implantation, scaffolds must be able to elicit a negligible immune response to avoid body rejection. Scaffolds should not be permanent for cells to produce their extracellular matrix (Dong & Lv, 2016). Hence, they must be biodegradable, non-toxic, and manage to leave the body without any disturbance (Mansouri, 2016; Zidan *et al.*, 2018). Now that this technique is getting interest in clinical practice, immunology plays a crucial role in this research area. Besides that, scaffolds should have consistent mechanical properties specific to their anatomical sites (O'brien, 2011). The cell porosity and mechanical properties must be complementary for cell infiltration and vascularisation, which is the critical component of a useful scaffold (Mansouri, 2016). Moreover, the architecture of the scaffolds used are of vital importance since they require interconnecting pore structures with increased porosity to allow nutrients diffusion for cellular growth (Mansouri, 2016). The pore size varies depending on the cell types and tissues engineered (Murphy *et al.*, 2010). The scaffold manufacturing should be cost-effective and reproducible for large scale applications, especially in clinical practices (Naahidi *et al.*, 2017). This is very important as it ensures continuous translational tissue engineering applications towards clinical fields.

COLLAGEN

Collagen is the primary structural protein of mammalian connective tissues that are ubiquitous in tendons, ligaments, bones, and skin (Purcel *et al.*, 2016). It plays a pivotal role in sustaining the biological and structural integrity of ECM that provides physical support for tissues (Guarino *et al.*, 2015). The early phylogenetic tree displays the presence of collagen was found in primitive marine animals such as jellyfishes, corals, and sea anemones (Purcel *et al.*, 2016). The fabrication of collagen-based materials in regenerative medicine has been used on skin, intestine, and wound dressing for more than a couple of thousand years ago (Meyer, 2019). Collagen and Matrigel are quintessential ECM applications for their remarkable biocompatibility and natural adhesive properties. These features support various physiological cell functions, resulting in enhanced cell viability, controlled proliferation, and differentiation, typically observed in an in vivo environment (Langhans, 2018). They stimulate cell adherent via integrin receptors that activates cell signaling pathway, which is essential for cell survivability, growth, and proliferation.

Nevertheless, the use of collagen in medicine began only half a decade ago following advancements in cleaning and sterilisation technologies (Ramshaw, 2016). The technology of collagen sterilisation brings a myriad of new applications in tissue engineering fields for instance, burn treatment, haemostasis, hernia repair, bone and cartilage defects, wound closure and dental practices (Copes *et al.*, 2019). In ophthalmology, the application of collagen receives spectacular responses in corneal shield, eye implantation for post-operative recovery, and corneal implantation (Eshar *et al.*, 2011; Zidan *et al.*, 2018). Due to rapid evolution of collagen techniques, recent development focuses on the cellularisation of tissue architecture for organ transplantation (Meyer, 2019). For example, a study conducted by Yeung *et al.* (2019) demonstrated that the microencapsulation of human osteoarthritic chondrocytes into collagen better recapitulated the osteoarthritis phenotypes. A recent study by Redmond *et al.* (2022) also indicated a highly biocompatible collagen scaffold, capable of facilitating the attachment and proliferation of MCF7 breast cancer cells over 2 weeks culture. Besides that, current progress focuses on the collagen-based bio-inks that are used for 3D bioprinting.

THE STRUCTURE OF COLLAGEN

Collagen constitutes relatively 25 to 35% of total mammalian body protein (Luo *et al.*, 2017). To date, there are 28 different types of collagens, and they are classified into four major groups according to their compositional and structural features (Arseni *et al.*, 2018). The trimeric structure of collagen has three polypeptide α chains that are woven into a triple helix, forming homotrimer or heterotrimeric molecules, as illustrated in Figure 2 (Liu *et al.*, 2015). Each trimer molecules have Gly-X-Y repeats in which X is proline and Y is 4-hydroxyproline (Sorushanova *et al.*, 2019). Each collagen has at least one triple-helical domain (COL) and non-collagenous (non-Gly-X-Y) domain (NC domain) that is located in the ECM. The NC domain assembles the collagen structures and endows them with biological activities (Luo *et al.*, 2017). The COL domain is scattered freely within the NC domain, forming multidomain proteins (Dong & Ly, 2016). Other main components that are involved in the formation of a triple helical structure are the C- and N-propeptides, a protein that is cleaved during protein activation or maturation (Purcel *et al.*, 2016). The C-propeptide is involved during the initiation of the triple helical structure meanwhile, the N-propeptide is responsible for the adjustment of primary fibrils diameter (Arseni *et al.*, 2018). The covalent bonding between the Gly and Proline preserves the twisted structure of the collagen, while the presence of 4-hydroxyproline is essential for the stability of the triple helix conformation (Purcel *et al.*, 2016).

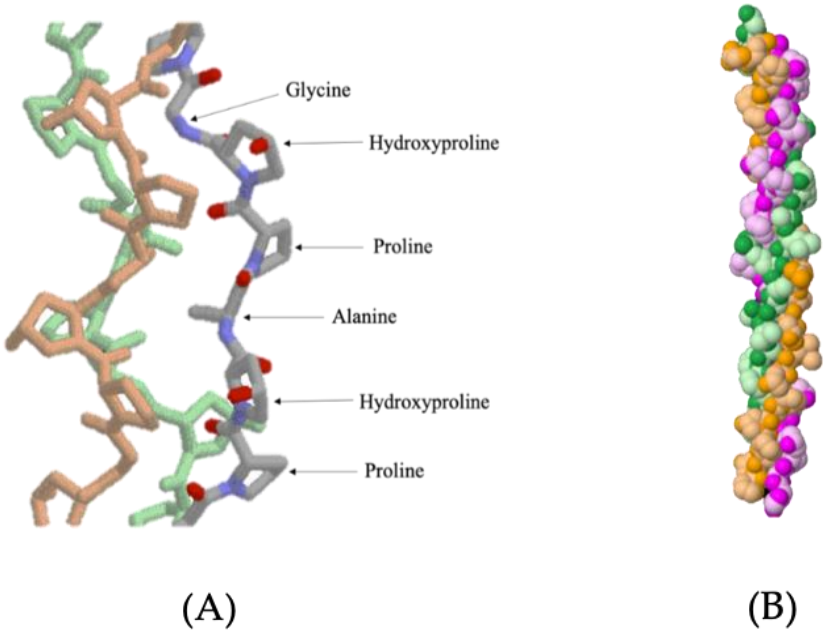

Figure 2. A classical view of the triple helix structure of the collagen. The image illustrates the collagen skeletal structure with Gly-X-Y repeats (A) and the 3D collagen molecular structure (B). Adapted from Bella *et al.* (2016).

SOURCES OF COLLAGEN

There are diverse sources of collagen that could be extracted from animals such as kangaroos, alligators, and marine species like sponges, jellyfish, and fish (Purcel *et al.*, 2016). Marine collagens are broadly used in the industry, but they are slightly utilised in research and clinical applications. Nevertheless, the conventional collagen extraction that is used in soft tissue applications nowadays originated from porcine and bovine skin, as well as rat tails (Purcel *et al.*, 2016). Tendons are preferably one of the ideal choices of collagen source due to their relatively high concentration of collagen I in contrast to other tissues (Rittié, 2017). It is practically easy to isolate with low material contamination. It is readily available and could be simply obtained from surgical remnants such as mouse or rat tails

in which, no ethical requirements are needed (Rittié, 2017). Apart from the tendon, collagen from the skin is also extensively used as it is mainly composed of non-collagenous proteins and lipids. However, additional steps are warranted during collagen purification as these proteins can cause material contamination (Rittié, 2017). Various ultrastructure studies focus on the rat tail tendon as a collagen source, although fibril diameters and distribution vary in terms of age, tissue, and stratified tissues (Raspanti *et al.*, 2018).

EXTRACTION AND STERILISATION

Collagen can be extracted through various experimental measures such as mechanical, physical, chemical, and enzymatic treatments. The extraction of collagen requires the removal of multiple intra- and intermolecular covalent crosslinks that consist of lysine and hydroxy-lysine residues, esters, and saccharides bonds (Ran & Wang, 2014). It was first discovered that the triple-helical collagen could be extracted using organic acids like acetic acid and citric acid. To date, the preparation of soluble collagen remains quite similar for every method (Ran & Wang, 2014). Collagen extraction is a time-consuming process due to the nature of the crosslinked structure present in the connective tissue of the animals (Schmidt *et al.*, 2016). The pre-treatment step is usually performed prior to collagen extraction to break the crosslinked structure and non-collagenous materials for higher yield. Chemical hydrolysis i.e. diluted acid or alkali is one of the pre-treatment approaches used to cleave the crosslinked structure and maintain the intact of the collagen chains (Prestes, 2013). This approach is more commonly used in the industry than enzymatic hydrolysis. Enzymatic hydrolysis involves the addition of enzymes such as pepsin, Alcalase®, and Flavoenzyme® that could enhance the nutritional value of a particular product (Moreira *et al.*, 2018). It reduces processing time for collagen extraction and generates less waste, but the cost is expensive (Schmidt *et al.*, 2016).

The crosslinking procedure is crucial to stabilise and enhance the mechanical strength of collagen fibres through natural, chemical, and physical methods (Gu *et al.*, 2019). Nature-derived crosslinking involves plant-based reagents like quinones, iridoids, oleuropein, secoiridoids, and cardanol (Meyer, 2019; Moreira *et al.*, 2018). Physical methods such as irradiation, dehydrothermal treatment (DHT), convection drying, solvent, and lyophilisation are performed to prevent the degradation of collagen fibres and the action of capillary forces through critical point drying (Persadmehr *et al.*, 2014). Lyophilisation is commonly used in sponge manufacturing as flat or cylindrical applications, especially in wound dressing and dentistry (Wang et al., 2019). On the other hand, the chemically derived crosslinking process is widely utilised in medical and pharmaceutical industries to stabilise collagen using isocyanates, carbodiimides, and bifunctional aldehydes (Wang *et al.*, 2019).

The necessity of sterilisation techniques for clinical and cell culture applications are vital to decontaminate the diverse sources of collagen. The extracted collagen should be free from all sorts of infectious organisms. They are temperature-sensitive hence, they are not autoclavable and require alternative sterilisation protocols (Delgado *et al.*, 2014). For example, filtration is used for low concentrations of acidic collagen solution (0.1-0.2%) that can be passed through 0.2 to 0.45 μm filters (Meyer, 2019). Radiation such as gamma (γ), ethylene oxide (b), and electron beam irradiations could also be used to sterilise the collagen (Shintani, 2017).

Nonetheless, the usage of these techniques may alter the molecular structure of the extracted collagen (Lalande *et al.*, 2019). For example, a low dosage of γ-ray has been found to alter the molecular structure which results in reduced mechanical strength and enzymatic resistance for the application of collagen scaffold (Harrell *et al.*, 2018). It requires additional glucose steps during irradiation to sustain the tensile strength of the collagen scaffold by the glucose crosslinking process

but it does not prevent structural degradation (Lalande *et al*., 2019). The combination of fungicide and antibiotics in ethanol is also acceptable for collagen sterilisation, even though it slightly altered collagen porosity in few grafts, such as porcine pericardium and in equine tendons (Delgado *et al*., 2014). Taken together, there are no perfect sterilisation techniques to date for collagen scaffold applications without structure degradation. There is a need to investigate the effect of sterilisation techniques on collagen materials so that the ultimate performance of the collagen-based biomaterials can be entirely understandable (Lida *et al*., 2020).

CONCLUSION AND FUTURE PERSPECTIVES

Over the last decades, 3D in vitro models have gained prominence, offering a significant improvement over traditional 2D culture systems. Collagen as scaffolds for ECM have emerged as key components in 3D culture, particularly in regenerative medicine as well as in oncology research. There are numerous types of isolation and sterilisation techniques available to produce functional and sterile collagen-based scaffolds for tissue engineering purposes. Each method has its specific advantages and limitations, depending on the intended application and the desired structural and functional properties of the scaffold. Future research should address the limitations of current collagen sterilisation methods to enhance scaffold performance and structural integrity. Innovations like bioprinting in collagen-based scaffolds hold great promise for more personalized tissue models. This model would be beneficial in drug discovery, especially in cancer research for its feature to study tumor growth dynamics, proliferation rates, and angiogenic characteristics.

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