Trends in Biotechnology

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Review

Advances in cell coculture membranes recapitulating *in vivo* microenvironments

Jin Yoo,[1] Youngmee Jung,[1,2] Kookheon Char,[3] and Yeongseon Jang[4,@,*]

Porous membranes play a critical role in *in vitro* heterogeneous cell coculture systems because they recapitulate the *in vivo* microenvironment to mediate physical and biochemical crosstalk between cells. While the conventionally available Transwell® system has been widely used for heterogeneous cell coculture, there are drawbacks to precise control over cell–cell interactions and separation for implantation. The size and numbers of the pores and the thickness of the porous membranes are crucial in determining the efficiency of paracrine signaling and direct junctions between cocultured cells, and significantly impact on the performance of heterogeneous cell cultures. These opportunities and challenges have motivated the design of advanced coculture platforms through improvement of the structural and functional properties of porous membranes.

Highlights

Advances in heterogeneous cell coculture systems using semi-permeable membranes have made it feasible to create *in vivo*-like microenvironments for a myriad of biomedical applications.

The development of *in vitro* cell coculture systems facilitates our understanding of cell–cell interactions in tumors and stem cell differentiation.

Engineered coculture membranes play a significant role in heterogeneous coculture systems for drug testing, tissue barrier modeling, and tissue engineering, to name a few.

Rational design and engineering of porous membranes will bridge the gap between *in vitro* experiments and *in vivo* applications.

Introduction to heterogeneous cell coculture systems

Every tissue in the body is composed of multiple cell types maintained by a heterogeneous cellular environment. Thus, the development of *in vitro* **cell coculture systems** (see Glossary) that recapitulate the *in vivo* **cellular microenvironment** is indispensable to advance our understanding of cell–cell communications regarding infectious diseases [1], drug toxicity and efficacy, immune response, and stem cell differentiation.

Different types of coculture strategies, including direct contact and indirect coculture techniques, have been adopted according to the purpose of the research [2,3]. Direct contact coculture refers to culturing two or more types of cells in the same cell culture container, which is convenient to operate [4]. This method preserves cell–cell linkages and brings cells closer, which enables mimicking the natural state in the body, thus maximizing cell–cell interactions through cell membranes and cell–cell junctions. However, it is hard to separate the cells following direct contact coculture, and this makes it difficult to explore molecular mechanisms in a heterogeneous cellular environment [5]. Especially in stem cell differentiation, the homogeneity of the collected cells should be strictly preserved for therapeutic applications as contamination with exogenous cells might result in serious complications such as immune rejection after *in vivo* transplantation [6].

The indirect contact coculture denotes two types of cells separated without direct contact whereas cytokine diffusion between containers is allowed [7,8]. This strategy mainly includes **porous membrane coculture**, conditioned medium coculture, and conditioned extracellular matrix (ECM) coculture (Figure 1) [9]. Among these methods, porous membrane coculture has been regarded as a versatile tool since it not only allows cytokine exchange but also can regulate direct cell–cell contact by controlling the size of the pores in the membranes [10]. In addition, cocultured cells on porous membranes can be easily adapted to microfluidic devices that enable exposure to physiological fluid shear stress or perfusion systems [11]. In porous membrane-supported coculture systems, the structural features and chemistry of the membranes are critical for coculture performance. For instance, in a **paracrine signaling** assay, the membrane should

[1]Center for Biomaterials, Biomedical Research Institute, Korea Institute of Science and Technology (KIST), Seoul 02792, Republic of Korea
[2]School of Electrical and Electronic Engineering, YU-KIST Institute, Yonsei University, Seoul 03722, Republic of Korea
[3]School of Chemical and Biological Engineering, Seoul National University, Seoul 08826, Republic of Korea
[4]Department of Chemical Engineering, University of Florida, Gainesville, FL 32611, USA

*Correspondence:
y.jang@ufl.edu (Y. Jang).
@Twitter: @JangUFChE

allow effective cytokine crosstalk between cocultured cells but prevent direct cell–cell contact and mixing of the different types of cells [12]. In an organ-on-a-chip device, multiple cell types are separated by porous layers that mimic the basal membranes of barrier tissues. Depending on the functions of the target tissue, the porous membranes used in organ-on-a-chip devices should be designed to recapitulate the characteristics of tissue barriers such as those of the gut epithelium, vasculature, lung, cornea, and liver. We highlight several heterogeneous cell coculture systems based on the porous membranes and their recent advances for biomedical applications.

Commercially available coculture porous membrane

Transwell® is a commercially available porous membrane for cell coculture [13–16]. A semipermeable membrane is attached to the Transwell insert, and hangs over a culture dish to establish two containers for an indirect coculture system. To increase the interaction between cocultured cells, different types of cells are seeded onto porous membranes and the bottom surface of inverted porous membranes, respectively (Figure 2A). This ensures the separation of the two cell types but retains their ability to interact through the pores of the membrane. Transwell coculture systems allow cytokines (but not the cells) to pass through the membrane as the average diameter of the membrane pores is <3 μm, typically 0.4 μm. To promote cell–cell interaction in Transwell systems, researchers have developed a both-side seeding technique in which one cell type is cultured on the apical (top) side of the Transwell insert and the other on the basolateral (bottom) side [17]. This technique has been generally adopted when more active cell–cell interactions are required, such as in a **tissue barrier model**, compared with the traditional indirect coculture system. In this case, according to the pore size of the membrane, the extent of cell–cell interaction ranges from paracrine signaling and physical contact to **transmigration**.

The Transwell system has been conventionally used as a standard, and a typical membrane of low porosity and ~10 μm thickness is prepared by the track-etched fabrication method (Figure 2B) [18]. However, previous studies have shown that Transwell membranes hinder sufficient cytokine signaling as well as physical contact between cocultured heterogeneous cells. Several researchers have therefore endeavored to develop new coculture membranes to resolve the limitations of commercial membranes and to invest in advanced functionality such as stretchability and thermoresponsiveness. Furthermore, engineering strategies have been developed to precisely control various parameters such as pore size, porosity, and thickness that affect the permeability and transport of biomolecules from one compartment to the other (Box 1).

This review covers newly developed porous membranes, ranging from fabrication methods to biomedical applications, that are used for advanced membrane-based cell coculture systems. Coculture system-based cell engineering strategies basically aim to mimic 3D cellular microenvironments as these are crucial for the study of immune responses, cancer metastasis, and drug screening. For example, testing drug toxicity or screening drugs in heterogeneous cell coculture systems that mimic *in vivo* 3D microenvironments can lead to more valid results. This strategy also applies to drug transport studies across tissue barrier models to verify drug efficacy. Furthermore, engineered coculture systems are generally adopted for stem cell differentiation and tissue engineering [4,19,20] as 3D heterogeneous cell structures are critical for these applications. Hence, in this review we categorize recent studies dealing with advanced porous coculture membranes into three major applications: drug testing, tissue barrier modeling, and tissue engineering.

Coculture membranes for drug testing

To bridge the gap between preclinical data and clinical trials of new drugs, there has been significant growth of *in vitro* physiological micromodels based on human cells. It is expected that a more sophisticated and biomimetic environment will provide more accurate information to estimate cell

Glossary

Cell coculture system: an engineering system used to study the interaction between the cocultured heterogeneous cell populations. Cell coculture systems have attracted attention from synthetic biologists and engineers who study and engineer complex multicellular synthetic systems including various tissues.
Cellular microenvironment: the local environment surrounding cells that contains physical and chemical signals that can directly or indirectly affect cell behaviors.
Paracrine signaling: a type of cellular communication in which cells produce diffusible signals to induce changes in adjacent cells. Paracrine factors secreted by a cell (i.e., signaling molecules) diffuse over a relatively short distance.
Porous membrane coculture: coculture of cells using a porous membrane that enables *in vitro* partitioning/division of the cell microenvironments while allowing physical and biochemical crosstalk between the cocultured cells.
Tissue barrier model: an *in vitro* model of a tissue barrier which establishes tissue compartmentalization and regulates organ homeostasis. Representative tissue barriers in the human body include skin, lung, gastrointestinal tract, kidney, endothelium, and the blood–brain barrier (BBB).
Transmigration: cells migrate across a tissue barrier to enter a different cell population.

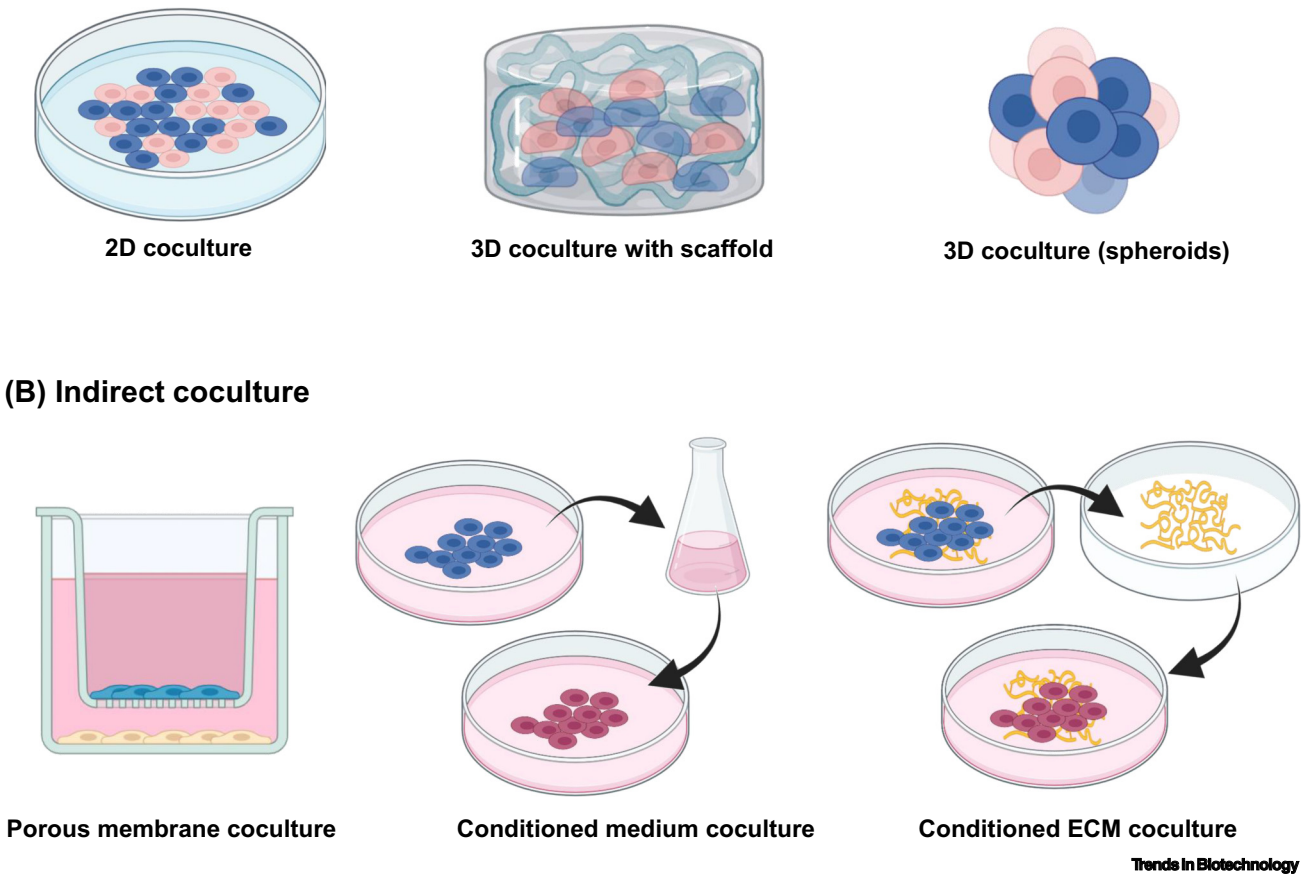

Figure 1. Different types of coculture systems. (A) Direct coculture models including 2D or 3D coculture platforms allow crosstalk between two types of cells via direct contact. (B) Indirect coculture models include porous membrane coculture (Transwell), conditioned medium coculture, and conditioned extracellular matrix (ECM) coculture that prevent direct contact between the two cell types.

responses during drug screening before clinical testing. Diverse coculture membrane-based engineering approaches have advanced towards more mature complex coculture models that aim to overcome the limitations of conventional systems. These include the development of coculture systems that mimic heterogeneous cell populations in tumors for screening anticancer drugs, thus enabling a reduction in both financial and time costs (Table 1) [21]. Tumors commonly encompass both genetically mutated and unmutated subpopulations of heterogeneous cell components. Broadly, the cellular stroma contains epithelial cells, normal and cancer-associated fibroblasts, endothelial cells, adipocyte cells, infiltrating immune cells, and pericytes, which assist cancer progression in various ways [22]. Thus, for studies on tumor progression and cancer treatment, it is critical to rationally design and control a more complex coculture model that accounts for the tumor microenvironment.

The development of transparent, nanoporous, and transferable (TNT) membranes is one example that has improved cell coculture systems to promote reciprocal communication between tumor and the stromal cells surrounding them, with increased cytokine transport efficiency than conventional membranes [12]. TNT membranes are prepared by nonsolvent vapor-induced phase

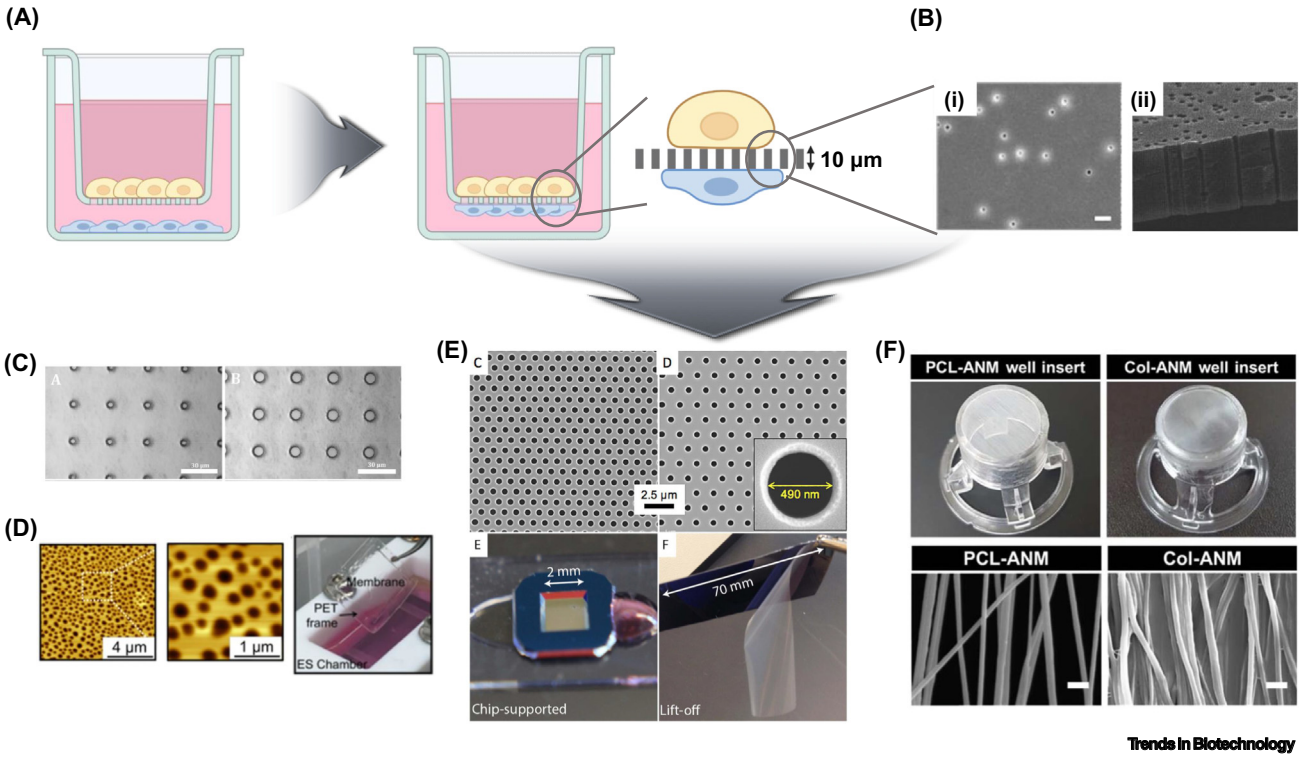

Figure 2. New strategies for coculture systems using porous membranes. (A) Schematic representation of the Transwell culture system. (Left) Indirect coculture system and (right) direct coculture system in which one cell type is seeded on the apical side and a second cell type is seeded on the underside of the insert. (B) (i) Surface morphology of commercial Transwell membrane [6]; scale bar, 2 μm. (ii) Side view of the membrane [18]. (C) Polydimethylsiloxane (PDMS) membrane [41], (D) poly(lactic-co-glycolic acid) (PLGA) membrane [48], (E) SiO_2 membrane [29], and (F) poly(ε-caprolactone) (PCL) electrospun membrane [34]; scale bars, 2 μm. Images in panels (B–F) are reproduced, with permission, from the indicated references. Abbreviations: ANM, aligned nanofiber membrane; Col, collagen-coated; ES, electrical stimulation; PET, polyethylene terephthalate.

separation of thin cellulose acetate films. The transparency, flexibility, and transferability of TNT membranes in cell culture medium originate from their nanometer-scale film thickness (480 nm) and small pore size (<150 nm). This membrane-based coculture platform has been used to mimic the tumor environment and study paracrine signaling between human breast metastatic cancer cells (MDA-MB-231) and three different types of stromal cells [human mesenchymal stem cells (hMSCs), NIH-3T3, and C2C12].

Another biocompatible and FDA-approved polymer, an eletrospun poly(lactic-co-glycolic acid) (PLGA) nanofiber membrane, has been utilized to establish an alveolar microenvironment [23]. The PLGA nanofiber membrane with a controlled thickness of ~3 μm allows good biocompatibility and permeability of molecules, and this facilitates its use as an alveolar respiratory membrane. Using engineered PLGA porous membranes, human non-small cell lung cancer cells (A549) and human fetal lung fibroblasts (HFL1) were cocultured to evaluate gefitinib, an epidermal growth factor receptor (EGFR)-targeted antitumor drug. The authors discovered the possible sources of A549 cell drug resistance in the presence of HFL1 cells [23].

The effective near-physiological tumor–vascular microenvironment was simulated by coculturing endothelial [human umbilical vein endothelial cells (HUVECs)] and fibroblast cells (NIH-3T3) on the different sides of a porous membrane, while breast tumor cells (MCF-7) were separately cultured in hydrogel [24]. A polycarbonate (PC) porous membrane (pore size 5 μm, thickness 10 μm) was

integrated into the microfluidic device to generate a vascular microenvironment. The system was used for an *in vitro* drug assay using the anticancer agent, doxorubicin, which confirmed that tumor cells respond differently to the drug when they are cultured in the *in vivo* mimetic microenvironment.

The side effects of anticancer drugs are life-threatening risks to cancer survivors, and cytotoxicity tests are therefore no less important than drug efficacy tests [25]. An improved 3D cellular configuration for *in vitro* drug toxicity evaluation has been developed by using multiple-layered cell sheets composed of cardiac-mimetic cells. Such multiple-layered, cocultured heterogeneous cells separated by nanoporous thin films help to evaluate drug toxicity *in vitro* and to investigate the effects of layer number on drug cytotoxicity outcomes [26]. For instance, thin nanoporous PLGA membranes prepared by spin coating were applied for direct cardiac reprogramming of human fibroblasts through coculture with cardiomyocytes and electrical stimulation. Double-layered sheets of human cardiac-mimetic cells were then built by stacking two layers of cardiac-mimetic cells on the PLGA membrane. Mono- and double-layered cardiac-mimetic cell sheets were treated with 5-fluorouracil (5-FU), an FDA-approved anticancer drug, to evaluate the toxicity of the drug. The cytotoxicity in the double-layered cell sheets was lower than that in the single-layered cardiac-mimetic cell sheet, which indicated that higher cellular interactions between cell sheets might contribute to inhibition of cytotoxicity.

Deng *et al.* developed a novel liver sinusoid-on-a-chip model to mimic the complex microenvironment in the liver sinusoid for hepatotoxicity assessment based on a prefusion liver system using a porous permeable membrane [27]. Two porous PC membranes with 1 μm pore size were used in the liver chip, and the hepatotoxicity of acetaminophen was measured. The authors also determined the variation in the hepatotoxicity of acetaminophen when a supplementary drug (rifampicin, omeprazole, or ciprofloxacin) was applied.

Box 1. Strategies for engineering porous coculture membranes

Several strategies have been developed to fabricate porous membranes for use in coculture systems, these include electrospinning, reactive ion etching (RIE), track etching, soft lithography, phase separation, and others (Figure I).

Electrospinning provides micro- and nano-fibrous structures that simulate ECM structures. Biocompatible/biodegradable natural or synthetic polymers are commonly used to prepare porous membranes, such as polycaprolactone, silk fibroin, and poly(lactide-co-glycolide). [23,34].

RIE and photolithography have been adopted to develop silicon-based porous membranes. SiMPore Inc. was founded to fabricate silicon and silicon nitride nanomembrane products for diverse biomedical applications [58–61]. Silicon dioxide (SiO_2) porous membranes are also made by the RIE process for cell coculture studies [33,62,63].

Track-etching technology is typically used to fabricate industrial and commercial porous membranes. Irradiation produces tracks in the foils and pore formation occurs via chemical etching [64]. Track-etched membranes offer advantages for controllable structures, such as pore size, shape, and density [65]. Track-etched membranes usually involve polycarbonate or polyethylene terephthalate.

Soft lithography is generally selected for the preparation of polydimethylsiloxane (PDMS) porous membranes. PDMS prepolymer is spin-coated onto patterned photoresist substrates and is peeled off from the surface following curing to fabricate PDMS porous membranes [66,67]. Transparent and elastic PDMS porous membranes are specifically used for organ-on-a-chip devices [68].

Phase separation/inversion can be used to introduce porosity into thin polymer films [69]. Phase separation of the polymer films exposed to nonsolvent in the vapor phase during spin coating results in ultrathin porous membranes that enhance cellular interaction between cocultured cells [12,47]. Of note, electrospinning and phase separation methods induce nonisotropic porous structures, whereas other methods lead to isoporous membranes.

In addition to the methods discussed earlier, 3D printing [51], breath figures [57], and selective leaching [53] have also been studied for the development of porous coculture membranes. 3D printing based on nozzle extrusion of polymer dissolved in solvent could provide porous membranes. The breath figure method is a solvent casting method that exploits high humidity conditions during film preparation. The pores are created by the condensation of water droplets onto the evaporating polymer solution, resulting in hexagonally packed pores. Furthermore, ultrathin porous membranes can be prepared by dispersion of binary polymer mixture on the aqueous interface, followed by selective etching of the polymer.

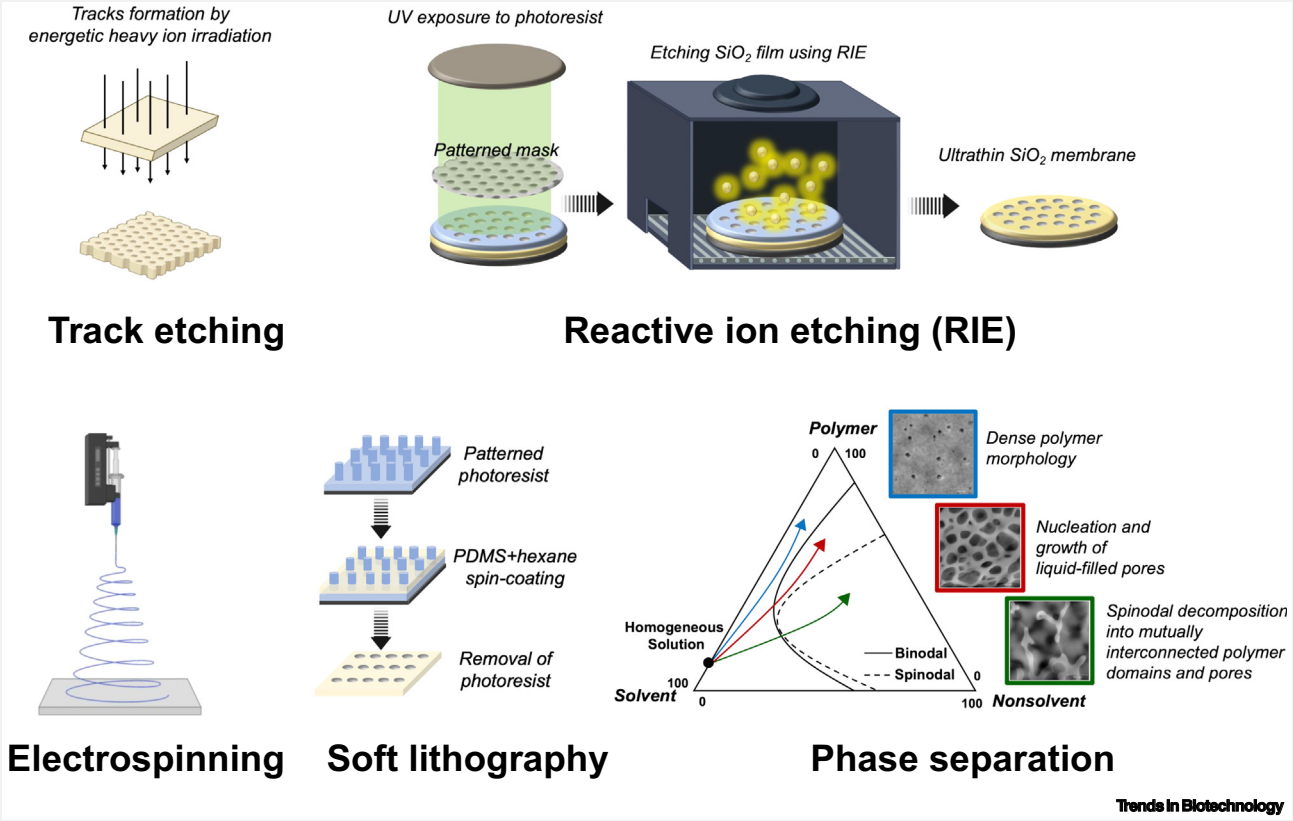

Figure I. Overview of the fabrication methods for porous membranes. Illustration of single-ion irradiation setup; adapted from [65]. Schematic representation of electrospinning and reactive ion etching (RIE) etching for fabricating silicon dioxide membranes; adapted from [29]. Fabrication process for a free-standing polydimethylsiloxane (PDMS) porous membrane using soft lithography; adapted from [70]. Phase diagram of hypothetical polymer, solvent, and nonsolvent system, and the resulting morphologies; adapted from [6,71]. The inset images to show morphology are reproduced, with permission, from [6].

These examples indicate the significance of advanced porous membranes for understanding cancer signaling and drug cytotoxicity testing. Track-etched PC membranes have the advantage of being easily integrated into microfluidic and perfusion systems owing to their robust mechanical

Table 1. Summary of membrane-based coculture applications for drug assessment

Drug type	Device and cell types	Material	Fabrication method	Membrane features	Refs
Gefitinib	Lung-on-a-chip Human non-small cell lung cancer cells (A549) Human fetal lung fibroblasts (HFL1)	PLGA	Electrospinning	Thickness ~3 μm	[23]
Acetaminophen, rifampicin, omeprazole, doxorubicin	Liver sinusoid-on-a-chip Four types of cell lines (HepG2, LX-2, EAhy926, U937) Tumor microenvironment (HepG2, MCF-7, HUVECs, NIH-3T3)	Polycarbonate (PC)	Track etching	Thickness 10 μm Pore size ~1 μm, 5 μm	[24,27]
5-Fluorouracil	Cell sheets of cardiac-mimetic cells [reprogrammed from human normal dermal fibroblasts (HNDFs)]	PLGA	Vapor-induced phase separation	Thickness ~540 nm Pore size ~300 nm	[26]

properties. Since they are commercially available, selection and control over the cocultured cell types and ratio becomes more practical for recapitulation of *in vivo* microenvironments for drug testing. However, the relatively high thickness and low pore density of PC membranes often limits effective signaling between cocultured cells, thereby demanding the development of new membranes such as TNT and PLGA. The newly designed membranes could sustain active cell–cell interactions through enhanced cytokine signaling because of their higher porosity and lower thickness. Collectively, sophisticated coculture systems combined with advances in porous coculture membranes can narrow the gap between preclinical studies and clinical trials in drug development.

Coculture membranes for tissue barrier modeling

The goal of an organ-on-a-chip device is to recapitulate tissue- and organ-level functions in a simple system [28]. Porous semi-permeable membranes, working as barrier models with defined apical and basolateral surfaces, are integral components for creating cellular or tissue interfaces in *in vitro* systems. There are several considerations in selecting a porous membrane for use as a barrier model in coculture systems, including pore size, film thickness, mechanical properties, and surface properties, to improve the physiological relevance and experimental control. Therefore, researchers have rationally designed and optimized the properties of porous membranes for tissue barrier modeling according to the purpose of use (Table 2).

Most track-etched, polymer membranes are fabricated with submicron-sized pore diameters which can prevent transmigration but hinder physical contact between cells cocultured on opposite sides of the membrane because the membrane is several micrometers in thickness. Carter and colleagues created an optically transparent ultrathin membrane with a porosity exceeding 20% and a thickness of ~300 nm, which is comparable in thickness with the vascular basement membrane [29]. The authors fabricated a tensile and robust porous silicon dioxide membrane by plasma-enhanced chemical vapor deposition of tetraethoxysilane. The ultrathin nature of the membrane facilitates the transfer of cytoplasmic cargo via gap junctions or extracellular vehicles between cocultured endothelial and adipose-derived stem cells through the membrane pores (pore sizes 0.5 μm and 3 μm).

The alveolar–capillary barrier constitutes a difficult challenge for the development of adequate *in vitro* models because the complex microenvironment of lung alveoli requires reproduction of cyclic mechanical stress induced by respiratory movements. Dohle and coworkers suggested an innovative bipolar cell culture model of the alveolar–capillary barrier containing microvascular endothelial cells, epithelial cells, and macrophages on a fully synthetic basement membrane [30]. They created a membrane with a thickness of 10 μm and an average pore diameter of 1.5 μm by using ultrathin nanofiber meshes (200 nm fiber diameter) electrospun from biocompatible poly(ε-caprolactone) (PCL).

Porous nanocrystalline silicon (pnc-Si) membranes were developed for the construction of tissue barrier models based on their ultrathin (30 nm), highly permeable, optically transparent, and biocompatible features [31]. Using pnc-Si nanomembranes, the transendothelial electrical resistance (TEER) of customized microfluidic systems was improved compared with conventional systems. Another endothelial barrier model was produced by fabricating an ultrathin (~1 μm) highly aligned, free-standing, PCL nanofiber membrane for use in microfluidic systems [32]. Coating the nanofiber membrane with Matrigel showed synergistic topographical and biochemical effects on the reconstitution of a well-aligned endothelial monolayer. Furthermore, a highly porous (~30%), dual-scale nano- and microporous silicon nitride (SiN) membrane, fabricated by a lithographic method, was shown to improve vascular transmigration [33]. The ultrathin (100 nm) and optically

Table 2. Summary of membrane-based coculture applications for tissue barrier modeling

Tissue barrier type	Cell types	Material	Fabrication method	Membrane features	Refs
Vascular barrier	Human umbilical vein endothelial cells (HUVECs), adipose-derived stem cells (ADSCs)	SiO_2	Pattern micropores with photoresist and etch film using reactive ion etching	Thickness ~300 nm Pore size ~0.5, 3 μm	[29]
Epithelial–stromal barrier/epithelial–endothelial barrier	Human bronchial epithelial cells (BEAS-2b), human lung fibroblasts (NHLFs)/BEAS-2b, HUVECs	ECM (collagen type I, Matrigel)	Cast ECM hydrogel and dehydration, followed by rehydration and crosslinking by transglutaminase	Thickness ~20 μm Pore size ~700 nm	[46]
Alveolar–capillary barrier	Human microvascular endothelial cell line (ISO-HAS01), human lung adenocarcinoma cell line (NCI H441)	PCL	Electrospinning	Thickness ~10 μm Pore size ~1.5 μm Porosity ~71 %	[30]
BBB	Human cerebral microvascular endothelial cells (hCMEC/D3), human astrocytes	PDMS	Patterning and PDMS etching	Thickness ~2 μm Pore size ~3 μm, 5 μm	[41]
BBB	hCMEC/D3, human astrocytes	SiN	Low-pressure chemical vapor deposition and RIE etching	Thickness ~400 nm Pore size ~0.5 μm Porosity ~20%	[40]
Physiological barrier	Bovine aortic endothelial cells (BAECs), HUVECs	Parylene	Plasma-enhanced chemical vapor deposition	Thickness ~300 nm to 1.5 μm Pore size ~3 μm Porosity ~25%	[56]
Blood–retina barrier	Human iPSC (hiPSC)-derived retinal pigment epithelium and endothelial cells	PLA, collagen	Breath figure method and collagen coating by Langmuir–Schaefer technology	Thickness ~10 μm Pore size ~3–6 μm	[57]
Endothelial barrier	Human mesenchymal stem cells (hMSCs), HUVECs	PLCL	Vapor-induced phase separation	Thickness ~960 nm Pore size ~1 μm	[44]

transparent dual-scale SiN membranes also enabled the creation of shear-primed endothelial barrier models. A collagen gel-coated and aligned PCL nanofiber membrane (thickness 4.5 μm) also offered enhanced endothelial barrier function [34]. HUVECs cultured on the membrane exhibited remarkably enhanced endothelial barrier function with high expression levels of intercellular junctions compared with the commercial Transwell models.

Natural polymers have been actively used for mimicking endothelial basement membranes owing to their biocompatibility and cell-interactive properties. Type I collagen is one of the most abundant proteins in the ECM and enhances cellular adhesion and response [35]. In this regard, a lyophilized type I collagen membrane was synthesized and incorporated into the microfluidic device [36]. Another study constructed artificial basement membranes through the layer-by-layer (LbL) assembly of the main components of natural basement membranes (e.g., collagen type IV and laminin) [37]. The multilayered nanofilms (thickness 5–80 nm) demonstrated the barrier effect of preventing cell migration but permitted effective cell–cell crosstalk between normal human dermal fibroblasts and endothelial cells (HUVECs), thus providing more reliable tissue models. Tibbe and colleagues fabricated membranes based on chitosan, a polysaccharide which forms a gel-like solid upon deprotonation [38]. This membrane (thickness ~80 μm) can be used as a physical barrier for cell culture, and the temporary membrane of chitosan can be removed by flushing with an acidic solution 24 h after cell seeding.

The blood–brain barrier (BBB) is an important system for the maintenance of central nervous system homeostasis. The most evident feature of the BBB is that tight junctions between brain microvascular endothelial cells inhibit the passage of many molecules by limiting paracellular permeability to an extent much greater than other endothelial cells. Several studies have endeavored

to mimic the permeability of this barrier. Bayir and coworkers selected bacterial cellulose (BC) as the basement membrane material for their *in vitro* BBB model [39]. BC can remain for a long time without degradation under cell culture conditions. In addition, BC is beneficial as a BBB model because its nanoporous structure (<200 nm) allows cell attachment but does not allow cells to transmigrate. TEER results demonstrated that the BC-based BBB model gives statistically higher transendothelial resistivity, thus showing its potential for use as a basement membrane for *in vitro* BBB modeling. Another study presented a BBB model that harnesses an ultrathin SiN membrane (0.5 μm pore size, 20% porosity, 400 nm thickness) integrated into a dual-chamber platform [40]. The platform includes human brain endothelial cells and primary astrocytes grown on opposite sides of the membrane. Moreover, an optically transparent polydimethylsiloxane (PDMS) membrane with 2 μm thickness was used for *in vitro* BBB modeling involving coculture of human cerebral microvascular endothelial cells and human astrocytes [41].

Membrane stiffness should also be considered, depending on the tissue. For instance, an elastic porous membrane is required for lung-on-a-chip devices to allow cyclic stretching. Most lung-on-a-chip devices use a thin, porous, and stretchable PDMS membrane [42]; however, they fail to recapitulate the characteristic alveolar network or the biochemical and physical properties of the alveolar basal membrane. Zamprogno and colleagues recently presented a lung-on-a-chip based on biological, stretchable, and biodegradable membranes (5–12 μm thickness) made of collagen and elastin that mimic *in vivo*-like dimensions [43]. Another study reported the development of elastic, porous, and ultrathin (~1 μm thickness) membranes that are stretchable [44]. The membrane was fabricated using poly(lactic-co-caprolactone) (PLCL) as the base material, where porosity is generated by vapor-induced phase separation. The stretched membranes induce the deformation of porous structures, leading to cell alignment, and these ultimately exhibited enhanced endothelial barrier function when hMSCs and HUVECs were cocultured.

Porous coculture membranes for establishing barrier models play a critical role in the *in vitro* realization of tissue barriers such as those of epithelia, endothelia, vasculature, lung, cornea, and BBB. Although 3D direct coculture models can simulate diverse types of tissue and their functions, they have limitations in studying transport and barrier properties [11]. Consequently, semi-permeable membranes are essential for studying tissue barriers because they not only need to act as a physical barrier creating a compartmentalized culture structure but they must also enable measurement of the transport and secretion of small molecules. Inorganic-based membranes (e.g., pnc-Si, SiO_2, SiN) are generally ultrathin and self-standing, enabling assembly into a cell culture device or chip. Furthermore, they provide optical transparency that offers direct *in situ* image analysis [45]. However, the use of rigid inorganic materials is not biofriendly compared with biocompatible/natural polymers. Thus, when an inorganic nanomembrane is used, coating with ECM materials (e.g., collagen and fibronectin) [40] is often performed before cell seeding. By contrast, polymeric coculture membranes comprising ECM materials are cell-friendly and imitate the basement membrane structure closely [37,46]. Nonetheless, some optimizations are necessary before polymer coculture membranes can be used as a tissue barrier model. If the membrane is ultrathin (<80 nm thickness), the membrane is not self-standing [37], which makes it difficult to integrate into devices or other cell culture systems. In the case of thicker free-standing polymeric membranes (>900 nm thickness), high-resolution imaging using optical microscopy is difficult because of light scattering and autofluorescence from the support materials [40,44]. Despite these shortcomings, polymer-based coculture membranes can be obtained at a large scale with relatively low cost, and their mechanical properties such as elasticity can be tuned by controlling the monomer ratio for copolymerization and/or the ratio of the curing agent.

Coculture membranes for tissue engineering

Finally, we highlight applications of newly developed coculture membranes for tissue regeneration and tissue engineering (Table 3). The induction of stem cell differentiation into the desired cell types before implantation is thought to be crucial for optimum therapeutic efficacy in stem cell therapy. Coculture of stem cells with the desired type of differentiated cells has been reported to be highly effective for controlling the fate of stem cells because it provides active cell-to-cell crosstalk. Nanothin and highly porous membranes, which are ~20-fold thinner and ~25-fold more porous than conventional Transwell membranes, were developed to achieve efficient stem cell differentiation [6]. The tunable thickness and pore size of the membranes allow control over the interactions between the cocultured cells (i.e., hMSCs and H9C2 cells). It was demonstrated that the nanothin and highly porous membranes were more effective in inducing stem cell differentiation and facilitating cardiac-differentiated cell sheets, and this was attributed to the thermoresponsive properties of the membrane. Another study presented a cellular LbL (cLbL) coculture platform using biodegradable, nanothin (~500 nm thickness), and highly porous PLGA membranes [47]. The cLbL coculture platform better mimicked the *in vivo* 3D microenvironment while also promoting cellular crosstalk between cocultured cells, and led to more efficient stem cell differentiation compared with conventional bilayer coculture systems. The cLbL coculture platform demonstrated augmented interactions between MSCs and chondrocytes, and showed enhanced chondrogenesis with suppressed hypertrophy of MSCs.

Song and coworkers introduced a cardiac-mimetic cell culture system that resembles the microenvironment in the heart and provides interactions with cardiomyocytes and electrical cues to cultured fibroblasts for direct cardiac reprogramming [48]. The authors cultured human neonatal dermal fibroblasts containing cardiac transcription factors on a porous PLGA membrane (500 nm thickness) with murine cardiomyocytes in the presence of electrical stimulation. Owing to active interactions between fibroblasts and cardiomyocytes cocultured on the PLGA membrane, the efficiency of cardiac reprogramming was dramatically enhanced. This platform was further advanced for cardiac repair by constructing prevascularized, multiple-layered cell sheets of direct cardiac reprogrammed cells [49]. This coculture-based tissue engineering method showed improvement in cardiac function and reduction in adverse cardiac remodeling post-myocardial infarction.

Another porous membrane-based coculture platform for cardiac tissue engineering was introduced by Suhaeri and coauthors [50]. A hybrid scaffold that combines aligned electrospun PLCL fibers and fibroblast-derived ECM (FDM) was proposed to induce effective cardiomyoblast

Table 3. Summary of membrane-based coculture applications for tissue engineering

Tissue type	Cell types	Material	Fabrication method	Membrane features	Refs
Skin tissue	Keratinocytes (HaCaT), fibroblasts (L929s)	Silk fibroin	Electrospinning	Fiber diameter ~0.88 μm Pore area ~5–147 μm^2	[52]
Retinal tissue	Human retinal pigment epithelial cell line (ARPE-19)	PCL	Drop casting of polymer blend on a liquid interface	Thickness ~9 μm Pore size ~100–200 nm	[53]
Cartilage tissue/cardiac tissue	hMSCs, rabbit chondrocytes/ reprogrammed HNDFs, murine cardiomyocyte cell line (HL-1)	PLGA	Vapor-induced phase separation	Thickness ~500 nm Pore size ~400 nm Porosity ~27 %	[47–49]
Cardiac tissue	H9c2 cardiomyoblasts, NIH-3T3	PLCL and fibroblast-derived ECM	Electrospinning and decellularization	Thickness ~30 μm Fiber diameter ~600–1160 nm	[50]
Bone tissue	Endothelial progenitor cells, human bone marrow stromal cells	PLA	3D printing	Thickness ~100 μm Pore size ~200 μm	[51]

(H9C2) differentiation and improve cardiomyocyte phenotype and maturation. Fibroblasts were cultured on PLCL nanofibers for 5–7 days and subsequently decellularized to obtain PLCL/FDM membranes. The coculture platforms demonstrated feasibility in terms of higher cell viability and ease of harvesting the target cells; importantly, there was a significant increase in cardiomyocyte phenotype and maturation markers compared with traditional coculture models.

3D-printed PLA porous membranes (100 μm thickness, 200 μm pore diameter) were used as scaffolds for bone tissue engineering [51]. Human bone marrow stromal cells and endothelial progenitor cells were cocultured in 3D using LbL assembly of PLA membranes. The results indicate that LbL assembly of cellularized PLA layers could be suitable for bone tissue engineering because they showed increased cell proliferation and osteoblastic differentiation. In addition, a custom-made scaffold was proposed for skin tissue engineering by coculture of keratinocytes (HaCaT) and fibroblast cells (L929) [52]. To be specific, an electrospun silk fibroin scaffold (fiber diameter 0.88 μm) was incorporated into the cell culture insert for dual cell seeding on either side of the insert. Subsequent coculture studies using the dual cell seeding approach revealed successful fabrication of a skin equivalent wherein HaCaT cells formed the epidermal equivalent and L929 cells formed the dermal equivalent.

Moreover, a free-standing PCL membrane with nanometer-sized pores is being investigated for mimicking Bruch's membrane, an ECM that acts as a molecular sieve to maintain metabolic exchange between the vasculature and the outer retina [53]. The membrane was prepared through drop casting of a polymer blend (PCL and PEG) on a liquid interface. It was demonstrated that the fabricated ultrathin and porous membranes can act as potential prosthetic Bruch's membrane for retinal tissue engineering.

Indeed, cellular interactions including direct cell–cell contact, cell–ECM interaction, and signaling via soluble factors support cells and promote tissue homeostasis, metabolism, growth, and repair. The types of cells within a coculture system for tissue engineering and regeneration are termed target cells and assisting cells. Generally, target cells are those that will eventually constitute the engineered tissue by reproducing the function of the target tissue, whereas the assisting cells guide the target cells to display a range of desired behaviors [54]. The goal is to generate tissues for implantation *in vivo*, where heterogeneous mixtures of cells can be formulated and engineered as a form of coculture. In this coculture system, when xenogeneic cells are used as assisting cells to generate the tissue, the cocultured cells must be separated to preserve the homogeneity of the collected cells [6]. Therefore, membrane-based coculture systems that allow cells to reside in environments similar to the native tissue, while retaining active communication with other cell types, provide benefits for tissue engineering.

Concluding remarks and future perspectives

The porous membrane-based coculture platform is crucial for cell research because it enables the creation of modular cellular and/or tissue interfaces and helps to establish barrier models for tissue-on-a-chip devices. New membranes with low thickness and high porosity have been developed to overcome the limitations of commercially available coculture membranes while also showing enhanced cell–cell interactions and mimicking the native tissue 3D environment. Intercellular signaling is a key component of diverse biological responses such as inflammation, tumor progression, and differentiation where two or more adjacent cell types simultaneously respond to an external cue. Collectively, the coculture platform is an indispensable tool to seek a basic understanding of cell–cell communication and also to study immune responses and disease progression. In this review we have categorized the applications into three fields

Outstanding questions

How can we create *in vitro* cell coculture systems that mimic *in vivo* microenvironments using porous membranes?

What membrane properties should be considered? In addition to biocompatibility, chemical stability, transport controllability, transparency, and extensibility, what other characteristics need to be investigated?

What properties of porous membranes should be prioritized to achieve optimum performance in cell coculture for drug testing, as tissue barrier models, or for tissue engineering?

Can we develop a coculture membrane with uniform pore size, good mechanical stability, biocompatibility, and low cost?

What new biocompatible polymers can be employed and functionalized for the preparation of coculture porous membranes?

Micro- and nanofiber membranes can simulate the ECM; however, because they are micro-thin, their ability to support themselves (i.e., self-standing properties) is compromised. How can we produce nanothin and fibrous membranes that mimic the ECM microenvironment while promoting transport?

Can we advance thin film fabrication methods to open more medical applications?

Table 4. Summary of the characteristics of porous coculture membranes according to the fabrication method

Fabrication method	Materials	Membrane features	Comment
Track etching	PC, PET	0.2–8.0 μm pores, 10–25 μm thickness, 0.08–44 cm^2 area, 10^5–10^8 pores/cm^2	Commercially available (Transwell®, Isopore™ membrane filter)
Photolithography and reactive ion etching	pnc-Si, SiO_2, SiN	10 nm to 3.0 μm pores, 15–400 nm thickness, 1.4–6.4 mm^2 area, 5–20% porosity	Commercially available from SiMPore Inc. TransparentD ifficult to obtain a large area
Electrospinning	PLGA, PCL, silk fibroin, PLCL	1.5 nm to 10 μm pores, 3–30 μm thickness, 50–70% porosity	Biocompatibility because of fibrous structureL arge area availableB iodegradabilityD ifficult to obtain images using optical microscopy because of opacity
Soft lithography	PDMS	2.0–10.0 μm pores, 2–10 μm thickness, 2–65% porosity	Large area availableT ransparent, elastic, and isoporous membrane Most widely used for organ-on-a-chip devices
Phase separation	Cellulose acetate, PLGA, PLCL	100 nm to 1.0 μm pores, 300–960 nm thickness, 20–50% porosity	Biodegradability, stretchability, and thermoresponsiveness may be added by selecting the material Large area available

(i.e., drug testing, tissue barrier modeling, and tissue engineering); however, these could be extended further [55].

The materials of the coculture membranes comprise polymers (i.e., cellulose acetate, PLGA, PDMS, etc.) and inorganics (SiN, SiO_2, etc.) (Table 4). Each material possesses different chemical and mechanical properties; researchers should therefore adopt optimized materials and conduct post-treatments such as surface coating or modification to create a cell-friendly environment [44]. The fabrication method should also be determined through consideration of cost and throughput (see Outstanding questions). For instance, drug screening requires high throughput and multiple repetitive experiments [21], which hinders the adoption of expensive coculture membranes.

Coculture systems using the newly developed porous membranes have recently been highlighted in biomaterials science and engineering for a range of biomedical applications. Nonetheless, commercial coculture membranes have set a standard in the field (although they are not a gold standard), leading to a knowledge gap between multidisciplinary fields in the area of advanced coculture systems. The overview presented in this article will therefore be of great interest to researchers aiming to develop artificial tissues. We also believe that advances in coculture systems with engineered, biomimetic porous membranes will narrow the gap between results from *in vitro* experiments and *in vivo* applications. Future investigations will be necessary to develop 3D heterogeneous cell coculture systems with precise control over membrane properties (e.g., transport of signaling molecules, mechanical stiffness, degradability, bio-affinity). Ultimately, interdisciplinary efforts in the rational design of porous membrane platforms for *in vitro* coculture and *in vivo* implants hold great promise in biotechnology and medicine.

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Declaration of interests

The authors declare no conflicts of interest.

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