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▶ To cite this version:

Guillaume Perry, Wenjin Xiao, Gavin Welsh, Adam Perriman, Rachel Lennon. Engineered basement membranes: from in vivo considerations to cell-based assays. Integrative biology, 2018, 11, pp.680-695. ⟨10.1039/C8IB00138C⟩. ⟨hal-01904479⟩

HAL Id: hal-01904479

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Engineered basement membranes: from *in vivo* considerations to cell-based assays

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Abstract

Improvements in the physiological relevance of cell-based assays have been enabled by the development of various interdisciplinary methods. However, due to their complexity, *in vivo* structures such as basement membranes (BMs), which regulate the phenotype of adherent cells, are still difficult to mimic *in vitro*. The reconstruction of a physiologically relevant BM is crucially important to develop cell-based assays with the capacity for drug screening and disease modelling. Here, we review the biophysical and biochemical properties of BMs *in vivo* and their interactions with neighbouring cells. We discuss the current methods used to mimic BM functions in cell-based assays according to the type of targeted applications. In doing so, we examine the advantages and limitations of each method as well as exploring approaches to improve the physiological relevance of engineered or cell-derived BMs *in vitro*.

I. Introduction

Since the invention of the petri dish in 1887 by Julius Richard Petri[1], there have been many improvements in cell culture approaches in order to reconstruct biologically and physiologically relevant cell-based assays, using interdisciplinary methods. These improvements have been achieved by the development of new technologies such as: (i) stem cells with both mesenchymal stem cells (MSCs)[2] and induced pluripotent stem cells (iPSCs)[3] (ii) microfluidics to control the cellular phenotype with chemical gradients[4,5] or shear stresses[6,7] and (iii) biomaterials, which regulate the microenvironmental cues.[8,9] However, these cell-based assays often lack relevant cell-extracellular matrix (ECM) interactions. This is particularly critical with assays aiming to recapitulate endothelial/epithelial tissues where cell phenotypes are regulated by the ECM from adjacent basement membranes (BMs).[10]

BMs are thin layers of specialized ECM proteins, which have various fundamental functions: (i) to provide a structural scaffold to support endothelial or epithelial cells, (ii) to act as a barrier between tissue compartments and (iii) to maintain neighbouring cell phenotypes.[11–13] These functions depend on the biophysical/biochemical properties of BMs, which change according to the localisation within the body.[14] In this review, we discuss the properties of *in vivo* BMs induced by the core BM components (type IV collagen, laminins, nidogens and heparan sulphate proteoglycans).[15–17] We highlight the impact of these properties on cellular phenotypes as well their modification due to disease.[18–20] We, subsequently, describe the different methods used to mimic the BM, mainly in coculture systems, according to their properties from polymer membranes to cell-derived ECM.[21–25] Finally, we conclude by comparing these methods according to the

targeted applications for cell-based assays, namely drug screening and disease modelling.[26,27]

II. *In vivo* basement membranes

In vivo BMs are very complex supramolecular assemblies formed by the interactions between two predominant networks of ECM proteins, type IV collagen (formed by three α chains) and laminin (formed by α, β and γ chains).[11,12] These networks are cross-linked by nidogens and interact with the heparan sulphate proteoglycans (*e.g.* agrin and perlecan).[28] Moreover, BMs are connected to cell surface receptors such as integrins or dystroglycans. [29–32] The BM molecular assembly is illustrated in **Figure 1**.[12,13,33] Besides the major components shown in **Figure 1**, various other molecules from the ECM are also present within the BM but in lower amounts, which makes it difficult to isolate and identify them. However, much recent progress has been made in this regard thanks to new "omics" technologies (genomics and proteomics) allowing for the characterisation of the matrisome.[34–36] Proteomic studies have now reported the complexity of: (i) the renal glomerular ECM composition, where the presence of 144 structural and regulatory proteins has been revealed [37], (ii) the ECM secreted by bone marrow-derived and adipose-derived MSCs [38], (iii) the ECM from decellularised rat liver [39] and others tissues.[40] This complexity also applies to BMs.[41]

a) Biophysical properties

An important biophysical characteristic of BMs is their thickness, which varies from 50 to 100nm according to their localisation, as well as their composition.[12,13] The mechanical stability of BMs is thought to be regulated by the type IV collagen network due to its unique structure and its abundance within BMs. This network is formed from a trimer of three α chains that bind together to form a triple helix and the trimers then assemble into

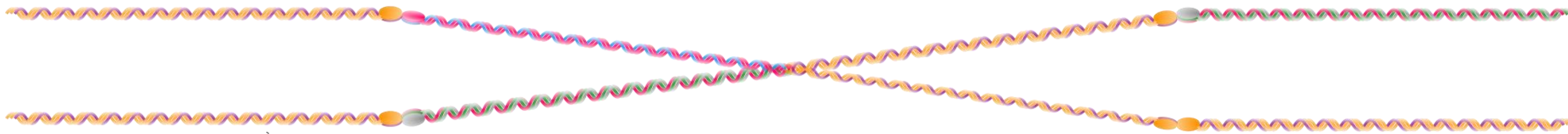
a network. The collagenous domains in the triple helix have hydrogen bonds and electrostatic interactions between the key residues (mainly glycine, proline and hydroxyproline) and these are likely to stabilize the molecule.[42–44] The two terminal domains, 7S domain at the N-terminus and the non-collagenous NC1 domain at the C-terminus also stabilize the network. This is achieved by the presence of cysteine and lysine residues, which crosslink the chains through disulphide bonds and lysine/hydroxylysine interactions at the N-terminus[45] and by non-covalent bonds mediated by various ions[46,47] and cross-linking formed via post-translational modification and sulphilimine bonds at the C-terminus.[48,49] Moreover, these cross-links also contribute to the mechanical properties of BMs. A recent publication by Bhave *et al.* demonstrated that type IV collagen sulphilimine cross-links in NC1 domain contribute to these nonlinear properties.[50] Moveover, these nonlinear properties are modified in peroxidasin knockout mice due to the reduction of sulphilimine cross-links. It is important to note that the stability of the type IV collagen network can be modified according to type IV collagen isoforms, particularly in the glomerular BM.[51] The independent laminin network also contributes to the BM stability because of its interactions with the neighbouring cells and its cross-linkage to the type IV collagen network with nidogen. Besides these two networks, the flexibility of the BM is also due the presence of perlecan, a heparan sulphate proteoglycan. This flexibility has been demonstrated to help the organ shaping in the drosophila.[52]

BM biophysical properties regulate the cell phenotype through various cues: the BM topography and the BM mechanical properties (*e.g.* stiffness, shear-strain response)[53], which depend on the BM structure. The BM topography is induced by the BM protein self-assembly, which generates a fibrous scaffold from type IV collagen and laminin networks.

This scaffold exhibits specific patterns in term of size and geometry at the nanometre scale.[54,55] As the previously described biophysical cues, these topographic features also depend on their localisation in the body and on the species as shown by *ex vivo* measurements.[53] Inspired by the topographic features found in native vascular endothelial BMs, Liliensiek and co-workers generated substrates with: (i) anisotropically ordered ridge and grove structures and (ii) isotropically ordered pores from 0.2μm to 2μm in size.[56] Using different human endothelial cell lines, they demonstrated that all cell lines exhibit a highly pronounced orientation and alignment on anisotropically ridges equal or above 0.8 μm in size. Moreover, one cell line (human umbilical vein endothelial cells) showed a decreased proliferation on the smallest topographic features. Along with this work, various reports have demonstrated that BM topographic features (size, geometry and isotropy) have a strong influence on the phenotypes of the overlying cells.[57–59]

Besides the BM topography, the BM stiffness or, in other terms, its elastic modulus (*i.e.* Young's modulus), plays an important role by regulating gene expression in response to mechanical cues by virtue of the links between the cytoskeleton and the ECM components via adhesion receptors.[7,60] It has been demonstrated *in vitro* that cell fate is influenced by the elastic modulus of the substrate on to which the cells adhere.[8,61] For this reason, cell behaviour is likely to be modulated by the Young's modulus of a BM, which varies from 1kPa to 2.57MPa according to the BM localisation.[53,62] Furthermore, the ECM components forming BMs exhibit nonlinear mechanical properties and a shear modulus ~100Pa.[63] Two particular properties have been described in the literature: (i) a nonlinear stress-stiffening observed on biological gels[64,65] and (ii) a nonlinear shear stress-softening observed on decellularised normal and fibrotic rat liver.[66] These examples of nonlinear

behaviour are typical of biological gels and cannot be reproduced using artificial polymers such as polyacrylamide. Furthermore, it also has been demonstrated that the BM mechanical properties strongly influence not only cell migration[67] but also BM breaching through mechanical processes.[68,69] Significantly, diseases targeting BM components can destabilize the BM structure (Table 1) and thus alter BM biophysical properties.



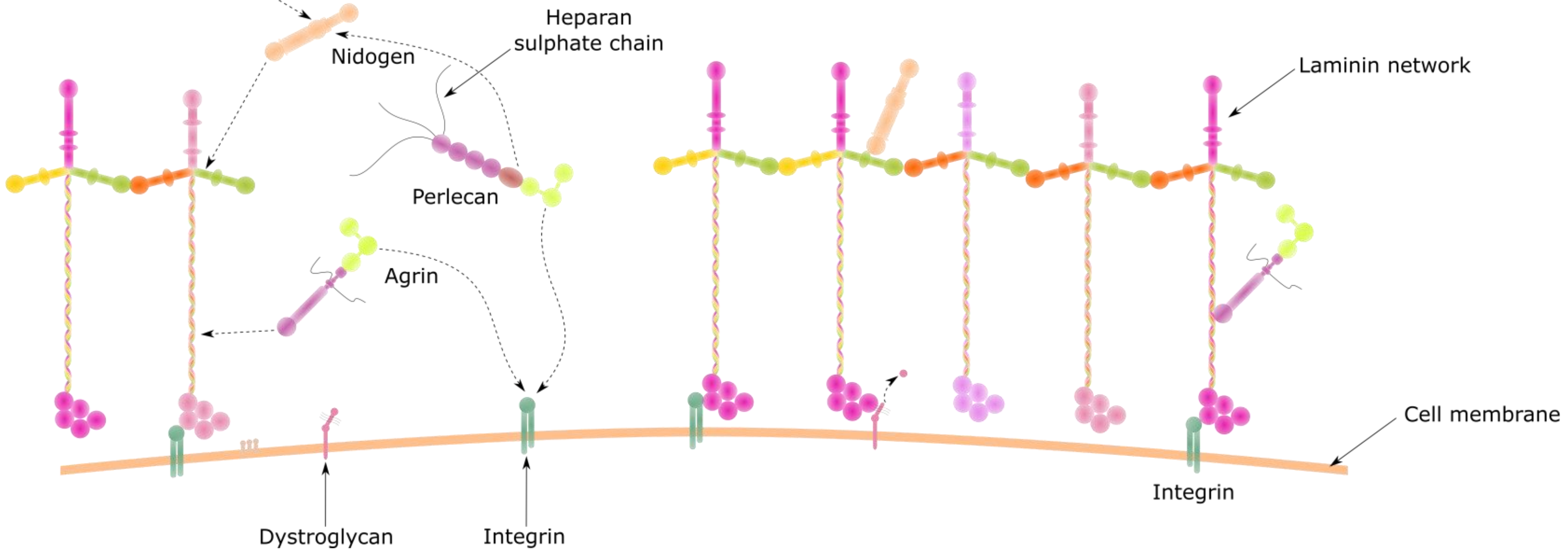

Type IV collagen network
Nidogen
Heparan
sulphate chain
Laminin network
Perlecan
Agrin
Cell membrane
Dystroglycan
Integrin
Integrin

Figure 1: Structure of the *in vivo* basement membrane composed of two predominant networks of type IV collagen and laminin, respectively. These networks are cross-linked by nidogen and interact directly or through agrin and perlecan, with the cell receptors (*e.g*. integrins and dytroglycan).

b) Biochemical properties

Along with these biophysical features, the biochemical properties of the BM are essential for cellular regulation due to the BM components. These properties are involved not only in various functions regulating neighbouring cells, such as cell adhesion and cell phenotype, but also in the diffusion of macromolecules or ions from the external environment and BM remodelling. As illustrated in **Figure 1**, cell adhesion on BMs is controlled by different types of biochemical interactions between the major cell surface adhesion receptors (*e.g.* integrins, dystroglycans) and matrix ligands. These adhesion interactions can activate intra-cellular signalling pathways leading to altered cell motility or cell differentiation.[70] Furthermore, it has been shown that laminin-integrin and laminin-dystroglycan binding are required to polarize the epiblast epithelium during early embryogenesis.[32] These interactions between the cell adhesion receptors and the laminin network explain the important role of laminin in numerous signalling pathways (modulation of growth factors, migration and inflammation of immune cells) that change cell phenotype.[29] The role of the laminin α subunits was studied during *C. elegans* development, showing that mutated α subunits disrupt BMs and lead to ectopic adhesion complex formation with downstream effects on cell-cell adhesion and cell signaling.[71] Furthermore, cell phenotype is also affected by various endogenous factors as BMs act as a reservoir for growth factors and release them according to biochemical stresses.[17] These factors include fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and Von Willebrand factor.[17] If laminin exhibits endothelial growth factor (EGF)-like domains, these growth factors are mainly

sequestered by the heparan sulphate proteoglycans (HSPGs). For example, perlecan is composed of 5 major domains with their own biochemical activities such as angiogenesis, growth factor and morphogen sequestration, lipid retention and cell surface binding.[72] Agrin is the dominant HSPG of the glomerular BM and plays an important role in neuromuscular junctions. Moreover, it contains domains that interact strongly with various growth factors.[37,73,74] Besides these GF-like domains, the HSPGs have also another function in the biochemical properties of BM due to their sulphated lateral chains. As a result of their negatively charged groups, they control the interaction with cationic proteins and positively charged ions (diffusion of molecules and ions through the BM). The loss of these lateral chains can impair various biochemical functions of the HSPGs, the filtration properties of the BM and be linked to diseases.[75,76]

Another important biochemical property arises from localized remodelling of the BM, which occurs during development, via controlled degradation to allow immune cells trafficking from the endothelium to the epithelium, and also during extravasation of metastatic cancer cells.[28,77,78] This disruption and remodelling are triggered by two families of metalloproteinases, which cleave the ECM components: (i) matrix metalloproteinase (MMP) and (ii) a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS).[79] Some fragments resulting from this degradation have been shown to have specific biochemical activities within the BM such as being anti-angiogenic (*e.g.* endorepellin)[17,72] or with other organs through the blood flow.[80–82] Furthermore, these biochemical properties depend strongly on the BM protein isoforms present within a particular tissue. The presence of the incorrect isoform or defects in the isoform structure can alter the BM properties as detailed in the **Table 1**.

Involved BM protein/chain in the disease	Effects on the BM biophysical properties	Effects on the BM biochemical properties
Type IV collagen α1[83]	Thin BM, disruption of the type IV collagen network	Loss of cell adhesion
Type IV collagen α3, α4, α5 chains[18]	Thin BM, disruption of the type IV collagen network	Loss of cell phenotype: (morphological change, high expression of matrix metalloproteinase)
Type IV collagen α3NC domain[44]	Disruption of the type IV collagen network	Activation of complement and proteases
Laminin β2 chain[84,85]	Defects in the laminin network	Loss of cell phenotype (morphological change, decrease in the expression of specific markers), modification of EGF-like domains
Laminin γ1 chain[86]	Defects in the laminin network	Modification of the EGF-like domains
Perlecan[87]	Modification of the BM flexibility,	Modification of the GF -like domains
Agrin[88]	Disruption of the binding with the laminin network	Disturbance of the biochemical activity in neuromuscal junctions

Nidogen-1[89]	Disruption of the binding between type IV collagen and laminin networks	Loss of cell phenotype (decrease glucose uptake, hypometabolism)

Table 1: Effects of the mutations in the basement membrane core proteins due to diseases, on the basement membrane biophysical and biochemical properties.

Although the conditions mentioned in **Table 1** are all rare diseases a range of therapies are currently in clinical use. In the case of Alport syndrome (mutations affecting type IV collagen $\alpha 3$, $\alpha 4$, $\alpha 5$ chains), angiotensin-converting-enzyme inhibitors are indicated from the point at which patients have persistent leakage of protein into the urine. [44] However, these drugs only slow the progression of the disease. In a recent review, Nystrom *et al.* proposed the development of cell therapy to restore permanently the damaged BM proteins in order to move forward to clinical applications.[90] The efficiency of such treatments could be tested within *in vitro* models before being translated to *in vivo*.

III. *In vitro* basement membranes

As described above, *in vivo* BMs are highly complex. Initial attempts to mimic these structures, were based on the use of simple polymer membranes but the complexity of *in vitro* membranes has been increasing over recent years.[91–94]

a) Polymer membranes

Due to their simplicity to fabricate by virtue of injection or soft-lithography, polymer membranes are widely used to mimic the *in vivo* BM within cell-based assays, particularly to test the BM permeability of the blood-brain barrier[95,96] or the glomerulus to drugs.[97] Different polymers: (i) polycarbonate, (ii) polyester, (iii) polyethylene terephthalate (PET) or (iv) polytetrafluoroethylene (PTFE) are used to generate these membranes and different porosities are commercially available to suit the targeted applications. These

membranes are attached to an insert in order to be compatible with cell culture well plates and to be used for permeability assay for drug screening as shown in **Figure 2a**. For example, Li *et al.* used a type IV collagen-coated PET microporous membrane to reproduce the glomerular filtration barrier by culturing podocytes and endothelial cells on each side of the membrane (**Figure 2a** D). They demonstrated that type IV collagen coating improved cell proliferation and phenotype compared to type I collagen coating.[97] However, this model does not fully reproduce the cellular microenvironment due to the lack of shear stress applied to the cells.

The integration of microfluidic functions within such polymer membrane-based assays led to the development of advanced cell-based assays, termed Organ-on-a-Chip, which show some promise for improving *in vitro* culture systems to mimic physiological functions. Furthermore, these assays use polydimethylsiloxane (PDMS), which is widely used due to its applications for rapid prototyping and microfluidics **(Figure 2b)**. These devices have been widely developed and used by research groups at the Wyss Institute.[21,98–100] Musah *et al*. recently generated a glomerulus-on-chip using podocytes derived from differentiated induced pluripotent stem cells (iPSCs) and microvascular endothelial cells separated by a PDMS membrane.[100] This coculture, assembled in a bespoke microfluidic chamber, was then used to study the permeability of the barrier in response to drug treatment. Despite the clear advantages of using an intervening membrane with fabrication and integration, there may not be a faithful mimic of the glomerular filtration barrier *in vivo***.** Moreover, these porous polymer membranes may not mimic the biophysical properties of *in vivo* BMs. The thickness of these membranes (1-10µm)[101] needs to be great enough to be handled easily during the fabrication/integration steps. However, the thickness used is considerably higher than

the thickness of *in vivo* BMs, and this may inhibit communications between the cells cultured on either side of the membrane. Cell-cell communication is also affected by the polymer membrane topography, which is limited by the pores generated during the polymer membrane fabrication. The porosity limits the use of this device in migration assays, as the polymer membrane cannot be degraded by the trafficking cells such as lymphocytes. Concerning the stiffness of the porous membrane systems, they generally present a Young's modulus (2.3GPa) three orders of magnitude higher than the highest *in vivo* BM Young's modulus (~2.57MPa).[62] This is also likely to influence cell phenotype.

To overcome some of these biophysical issues (BM thickness and integration), Pensabene *et al.* recently demonstrated a new method to generate an ultrathin polymer membrane (~100nm) in poly-L-lactic acid with pattern micropores (Ø≈2μm) within a microfluidic device.[25] They cultured human umbilical vein endothelial cells for two days, which attached onto the polymer membrane and displayed a good viability and spreading. Nevertheless, this ultrathin membrane does not exhibit *in vivo*-like topography and the Young's modulus was not investigated. However, polymer membranes can display topographic patterns generated at the micro/nanoscale using hot-embossing or soft-lithography to mimic *in vivo* ones.[56,102] One way to generate membranes with physiologically relevant Young's modulus is to use a different PDMS monomer and curing agent composition. Using this approach, it is possible to decrease the PDMS membrane Young's modulus from 1kPa to 3MPa[103,104], which is similar to *in vivo* BM Young's modulus. Although PDMS does not exhibit the same nonlinear strain-stress behaviour than *in vivo* BMs[103,105], the strain applied to the membranes within Organ-on-Chip improves to cell phenotype compared to those without.[21,100] This result suggest that the cultured cells might be mainly affected by the mechanical properties of the BM coating

and not those of PDMS. The biochemical properties of these polymer membranes are often linked to the ECM components (type I or type IV collagen, BM extract such as Matrigel) used to functionalise them. Hong *et al.* recently proposed a novel method to control the coating on the membrane within a microfluidic device.[106] Firstly, they cultured NIH/3T3 flibroblasts on the polymer membrane in order to generate fibroblast-derived ECM coating before removing the fibroblasts. However, the decellularisation process seems to damage the secreted laminin network and so alters the biochemical properties of the coating.

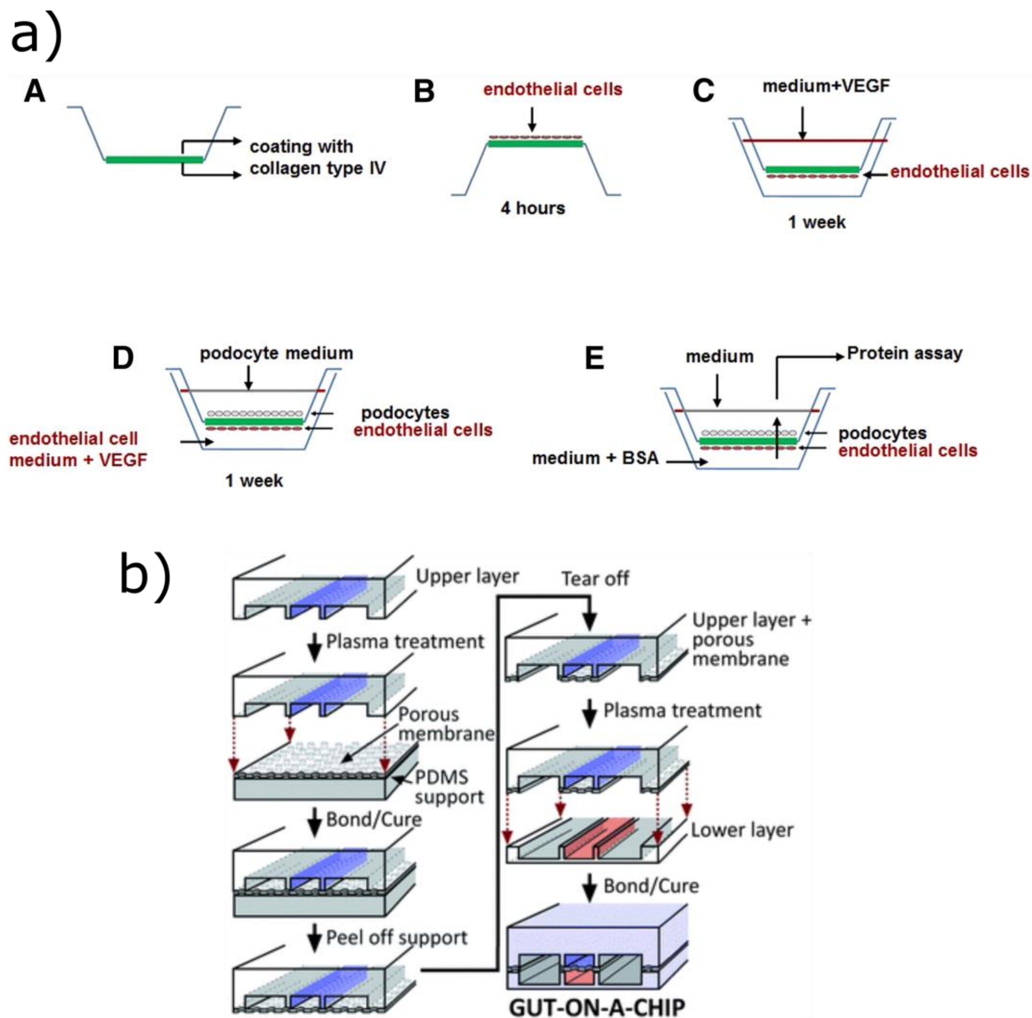


Figure 2: Different methods to use porous polymer membrane to mimic basement membrane. a) ECM-coated membrane insert to reproduce the glomerular filtration

barrier. Reproduced from Ref. 97 with the permission from Elsevier. b) Integration of porous polymer membrane (PDMS here) within a microfluidic device to generate a Gut-on-a-Chip. Reproduced from Ref. 98 with the permission from the Royal Society of Chemistry.

b) Electrospun scaffolds

Another method to generate BM-mimicking membranes is based on polymer extrusion due to an applied electric field between a needle and a metallic foil. This fabrication method has been used to create a different kind of membrane, electrospun scaffold with different materials. The main interest of this method is in the generation of a network of polymer microfibers (Ø≈1µm), which results in a higher porosity and higher surface area compared to the polymer membranes described in the previous section. The polymer mainly used in this application is polycaprolactone (PCL), due to its biocompatibility and its chemical and physical properties, and different kinds of biomolecules are employed in order to improve cell adhesion. All these methods showed a good cell adhesion and an improved cell phenotype due to the functionalisation with peptides or proteins. Slater *et al.* generated a PCL scaffold on a Ni mesh with cross-linked type I collagen nanofibers in order to create a physiologically relevant *in vitro* model of the glomerulus to study its filtration properties in presence of drugs or disease modifiers.[22] They cultured human conditional immortalized glomerular cells (podocytes and glomerular endothelial cells (GEnCs)) on each side of their scaffold. Although they did not investigate the mechanical and biochemical properties of their coated scaffold, the scanning electron microscope (SEM) images (**Figure 3**a) show a microfiber network on which the glomerular cells are lying and displaying their particular morphology and phenotype by expressing the

specific markers, podocin and PECAM-1 for podocytes and GEnCs respectively. Lv *et al.*[107] generated various scaffolds using PCL, silk fibroin and PCL/silk fibroin. The topography of these scaffolds with nanofibers are close to *in vivo* BMs as are the Young's modulus of these scaffolds. To coat their scaffold, they chemically extracted some BM proteins from porcine mucosal tissue and coated their PCL/silk fibroin scaffold with this extract in order to enhance epithelial regeneration. Although they showed that the BM protein coated scaffold had the best results in term of cell adhesion, proliferation and phenotype, they did not investigate in detail the contents of this extract. Recently, Ravikrishnan *et al.* developed an electrospun PCL scaffold on which they covalently cross-linked an RGD peptide to study the epithelial-to-mesenchymal transition of Madin-Darby canine kidney cells. The scaffold they generated, exhibited a good topography to reproduce the BM. However, they did not characterize the biophysical properties of the scaffold and its biochemical properties were just defined by the cross-linked RGD peptide. Rossi *et al.*[24] developed a novel method to reconstruct a skin culture model that could be used to study drug penetration through the dermis. They engineered: (i) a star-shaped polyether prepolymer with ethylene glycol and propylene glycol which was statically copolymerized with reactive isocynate groups NCO-sP(EO-stat-PO), and added to poly(D,L-lactide-co-glycolide) (PLGA) before the electrospinning and (ii) different peptide sequences from fibronectin, type IV collagen, α1-chain of laminin and β1-chain of laminin. As shown in **Figure 3b)**, the addition of the star-shaped polyether prepolymer helped to stabilize the electrospun scaffold compared to PLGA scaffold. Moreover, due to the reactive isocynate groups and the cysteine group present at the beginning of each peptide sequence, the peptide can be efficiently immobilised on the scaffold. The combination of these four peptides displayed the best performance in terms of cell proliferation, phenotype and secretion of ECM proteins. Compared to polymer

membranes, electrospun scaffolds are more physiologically relevant due to their fibrous aspects and their porosity. They also display a Young's modulus (10-35MPa)[24,107] one order of magnitude higher than the stiffness of *in vivo* BMs.[62] However, the investigation of the strain-stress properties of electrospun scaffold shows that they do not display nonlinear stress-strain properties such as *in vivo* BMs and exhibit a higher shear modulus (~1MPa).[107–109] Although they do not display specific biochemical properties, they can be easily coated by BM proteins such as the polymer membrane. Nevertheless, the presence of polymer in electrospun scaffolds limits their application because of polymer degradation.

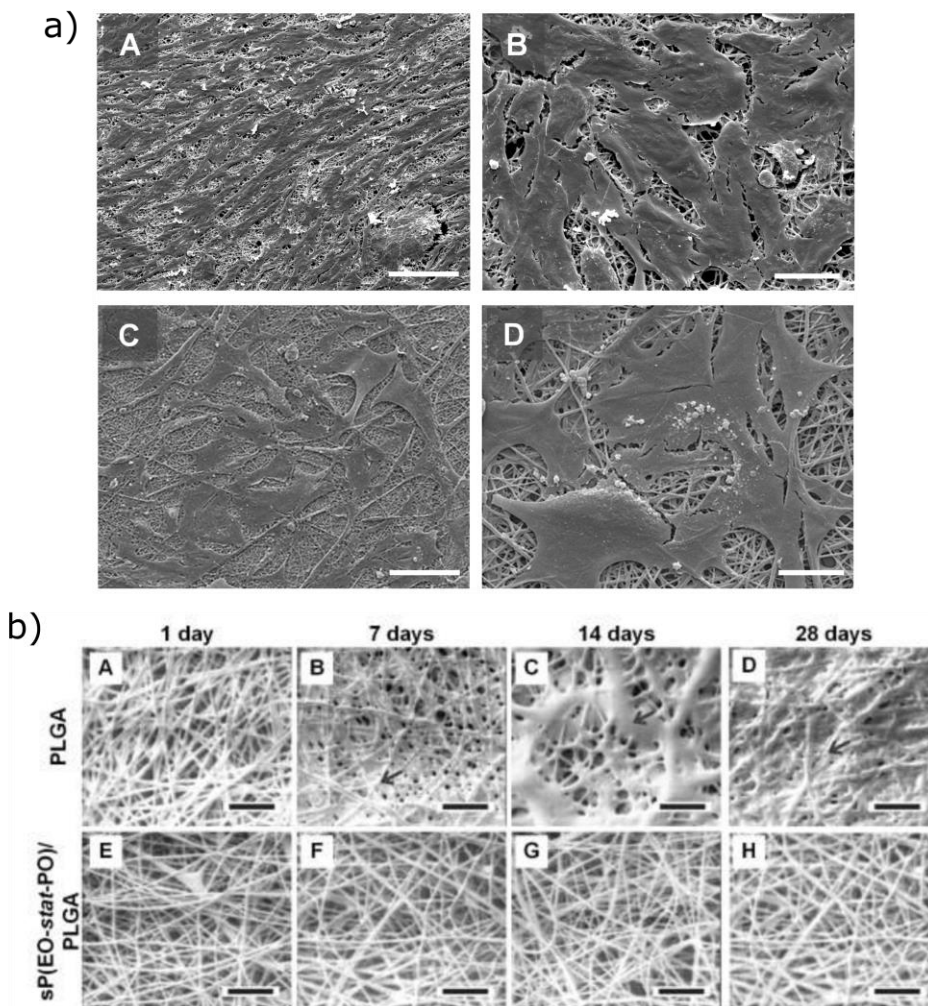


Figure 3: SEM images of: a) glomerular endothelial cells (A-B) and podocytes (C-D) cocultured on opposite sides of the collagen/PCL electrospun scaffold, scale bars=100μm (A-C), 20μm (B-D).

Reproduced from Ref. 22 under Creative Commons Attribution Licence. b) PLGA and functionalized PLGA electrospun scaffolds after 1 to 28 days in physiological buffer at 37°C, scale bars=5µm. Reproduced from Ref. 24 with the permission from Wiley.

c) Peptide/polysaccharide matrices

Different peptide/polysaccharide matrices were developed by the team led by Nomizu,[110,111] in order to reproduce BMs. They focused on laminin as it plays a major role in cell maintenance. They firstly screened various peptides from 12 amino-acids sequences of laminin-111, which promote cell adhesion and identified the other biological activities of these peptides already reported in the literature. To reconstruct the physical properties of the BM, they used two natural polysaccharides: chitosan and alginate, which do not initiate immune responses. The polysaccharides are chemically activated to covalently bind the peptides thanks to their cysteine residue at the N-terminus. The active peptide sequence is separated from the N-terminus by two glycine residues. They demonstrated that the amount of polysaccharides influences the biological activities of the immobilized peptides. They then altered the concentration of two peptides (AG73/EF1), to identify which promoted the best cell adhesion independently of the polysaccharides amount. They showed that a particular ratio of these two peptides is critical to get a synergistic effect to improve cell attachment and spreading. Compared to previous methods, this approach allowed a reconstruction of a BM without any polymer but with polysaccharides and laminin-based peptides. Although this method is interesting in that it avoids the use of polymers and is easily engineered, it does not accurately reflect the *in vivo* BM because of its biochemical properties, due to the lack of other BM components. Even if the biophysical properties of these alginate/chitosan matrices were

not characterized, the literature shows the possibility of engineering a wide Young's modulus range (1kPa-6MPa) and a highly porous structure, which is not fibre-like.[112,113] These matrices are useful to promote cell adhesion and phenotype in tissue engineering for transplantation.

A polysaccharide matrix was also recently used by Tibbe *et al.* to generate a self-standing chitosan membrane within a microfluidic device in order to reproduce the basement membrane.[114] Here, they formed a chitosan membrane by playing on the pH according to the method developed by Luo *et al.*, who studied the permeability of their membrane with fluorescein, labelled antibodies and labelled nanospheres.[115] Tibbe *et al.* then coated the membrane with Matrigel and seeded astrocytes on one side of the membrane. After 18h, they removed the chitosan membrane using a solution of acetic acid and seeded brain microvascular endothelial cells on the side of the membrane without cells. They showed that the astrocyte morphology is not altered by the membrane removal. The main advantage of this method is its direct integration within a microfluidic device, which can help to maintain the sterility of the device. To conclude on these polysaccharide matrices, they present various interesting properties such as their tuneable Young's modulus, their natural origin and their ability to be functionalised.

d) Extracellular matrix protein gel

This method is widely used in cell-based assays, as it is the one of simplest ways to mimic the BM *in vitro*. It consists of a layer of extracellular matrix protein between the two cell types cultured in the well plate. It was introduced in 1989 by Dunn *et al.*, who cultured primary rat hepatocytes between two thick layers from a type I collagen gel in order to reproduce the hepatic basement membrane and enhance hepatocytes polarization.[116] They were able to culture the primary cells for 42 days with a good normalized albumin

secretion but the total amount of DNA decreases by two times between the beginning and the end of the culture. This pioneering work suffers from several limitations such as the collagen gel thickness restricting the nutrient diffusion to the cells and the gel composition consisting of only type I collagen, which is not a major component of the *in vivo* BM. In parallel to this work, at the end of the 1980s, a number of groups, led by Timpl and Martin, attempted to understand the biochemical effects of the BM components and their interactions with the neighbouring cells.[15,117–120] This work led to a better understanding of the complex composition and structure of the BM and Kleinman *et al.*[118] developed a gel made of BM proteins from Engelbreth-Holm-Swarm murine sarcoma, which was studied by Orkin *et al.*[119] This gel, now known as Matrigel, is composed of type IV collagen (α1α1α2 isoform), laminin (α1β1γ1 isoform), nidogens, heparan sulphate proteoglycans and a number of growth factors such as FGF, EGF, TGFβ, IGF and PDGF (it also exists with reduced growth factors).[121] Matrigel has been used to reconstruct the BM and to increase the relevance of hepatocyte culture[122,123], mammary cells[124,125], prostate cells[126] or cancer cells.[121] Arends *et al*. used different commercially available BM protein gels and investigated the effects of their biochemical properties on their biophysical properties.[127] They demonstrated not only that the composition of these gels especially the nidogen concentration, is very different in each gel according to the manufacturer process but also that the gel composition affects its topography and its Young's modulus. Furthermore, they showed that the cell migration (human promyelocytic leukemia cell line HL-60) in the gel depends on its composition. BM protein gels like Matrigel are actually one of the best in reproducing the BM in cell-based assays as it is composed of only biomolecules but like the other methods described above it suffers from a couple of disadvantages. Firstly, due to its tumorigenic origin, its components and their concentration cannot reproduce accurately the desired BM and they display batch-to-

batch variation. Secondly due to its physical state, it is difficult to integrate them in advanced cell-based assays such as organs-on-chip without polymer membranes.[10] To overcome this integration issue, Takezawa and co-authors proposed a novel scaffold of type I collagen vitrigel, which is maintained by a silk fibre network or a nylon membrane ring[128], in order to reconstruct the BM of various organs such as the kidney[129], the liver[130] and the eyes.[131] Toh *et al.* reconstructed a collagen barrier to mimic the basement membrane within a microfluidic device.[132] They studied the migration of breast cancer cells (MX-1) through collagen barrier although without further characterisation.

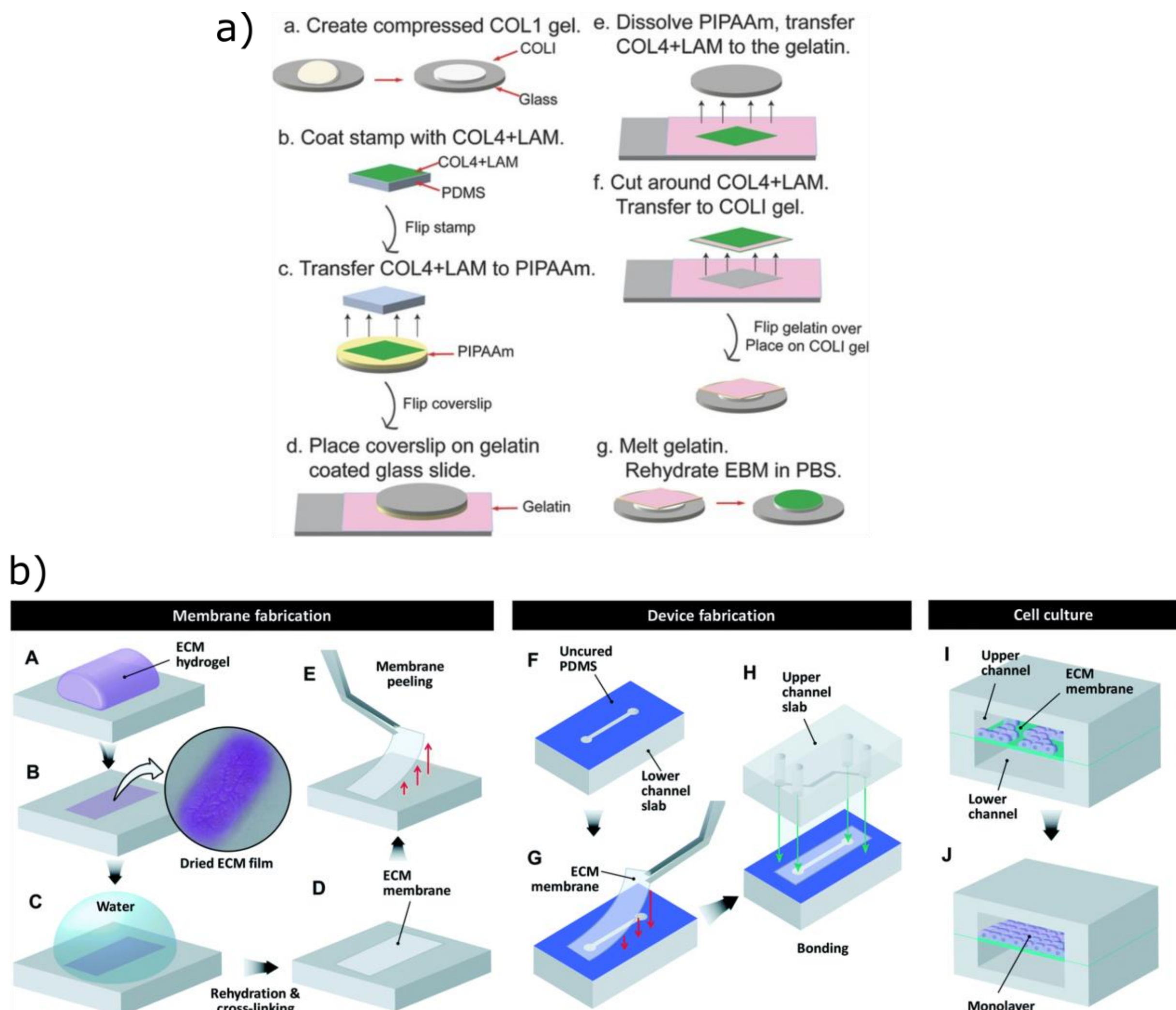


Figure 4 : Different methods to generate an extracellular matrix gel-based basement membrane: a) Reproduction of the Descemet's membrane with a thin type IV collagen/laminin membrane and a thick type I collagen membrane. Reproduced from Ref.

133 with permission from Wiley. b) Generation and integration of an extracellular matrix membrane within a microfluidic device for Organ-on-Chip applications. Reproduced from Ref. 27 with permission from the Royal Society of Chemistry.

More recently, Palchesko *et al.* proposed a novel engineered BM composed of type I collagen, type IV collagen and laminin to replace the Descemet's membrane in the corneal endothelium regeneration.[133] This membrane whose fabrication process is described in **Figure 4a**), exhibits a fibrous aspect according to the atomic force microscopy analysis. They showed that this membrane promotes cell adhesion, cell density and cell-cell tight junctions. Interestingly, they demonstrated that the cells cultured on this membrane secrete a laminin isoform, which does not match with the isoform initially present in the membrane. Despite its thickness and its unknown Young's modulus, this membrane provides a good material to reproduce a few of the properties of *in vivo* BMs (topography, cell phenotype).

Mondrinos *et al.* were the first to design a membrane made of only ECM proteins, which can be integrated within a microfluidic device (**Figure 4b**).[27] They studied the physical properties of the membrane and its effects on the cellular phenotypes using different mixtures of ECM components: (i) type I collagen, (ii) type I collagen and Matrigel or (iii) type I collagen and alginate by using a PDMS stab. They found with scanning electron microscopy that the membranes look like randomly oriented fibres forming a dense 3-D scaffold. They measured the thickness of their membrane (~20μm) and the Young's modulus of their membrane to compare with *in vivo* BMs. The Young's modulus of these membranes can be tuned according to type I collagen/Matrigel ratio from 429 to 660kPa. However, they did not investigate if this ECM blend exhibit a stress-stiffening property as type I collagen display one but not Matrigel.[63] To generate an Organ-on-Chip device, they

peeled off their membrane from the PDMS stab to insert it within a microfluidic chamber. They showed, using a permeability assay that the main difference between the ECM-made membranes is due to the presence of alginate creating larger pores. The permeability of 20kDa FITC-dextran of the collagen/alginate membrane is similar to a transwell membrane and higher than the collagen and collagen/Matrigel membranes. To assess the phenotype of cells cultured on the ECM membrane, they analysed the phosphorylation of focal adhesion kinase (pFAK) in human umbilical vein endothelial cells (HUVECs). The pFAK levels in the case of collagen/Matrigel membrane were more than nine times higher than the levels seen for the transwell membrane. Furthermore, they were able to culture different cell types, exhibiting a good viability, within the microfluidic device to mimic a lung-on-chip device. More recently, Humayun *et al.* proposed a novel method to generate an *in vitro* BM within a microfluidic device using a suspended hydrogel based on type I collagen and Matrigel mixture.[134] Interestingly, they demonstrated that the mixture with the highest Matrigel concentration give the best results in term of cell adhesion.

These latest works pave the way for further integration of a constructed representative BM within a microfluidic organs-on-chip by reproducing a good model of the BM without any polymer. However, there are still several drawbacks:

(i) Even though this membrane is only made of ECM components, it still does not accurately reflect the composition of *in vivo* BMs as type I collagen is a major component of this *in vitro* BM and Matrigel contains only some isoforms as previously mentioned.

(ii) The membrane thickness (~20μm) is higher than those *in vivo*.

(iii) Due its thickness, the membrane needs to be very carefully peeled off from the PDMS stub and inserted within the microfluidic device.

e) Layer-by-layer coculture

The easiest method to reproduce the relevant BMs relies on the secretion of ECM components by the layer-by-layer cocultured cells. As the different cell types secrete different components of the BM[135], various combinations of cells have been cultured such as keratinocytes/fibroblasts[136], Sertoli cells/Testis peritubular cells[137], Caco-2/intestinal mesenchymal cells[138], hepatocytes/liver endothelial[23] and podocytes/glomerular endothelial cells[139] without any layer between the two cell layer**.** The beneficial effect of coculture on the BM formation has been also proved when hepatocytes are cultured with liver endothelial cells (**Figure 5a**), especially when the cell oxygenation is well perfomed.[23] This coculture enhances cell phenotype through the expression of specific markers and the formation of bile canaliculi and keeps a proper cell viability over two weeks. Although it is possible to assess BM formation using histochemistry, it has also been demonstrated using immunofluorescence staining to show the different components of the BM (mainly type IV collagen or laminin isoforms).[138,139]

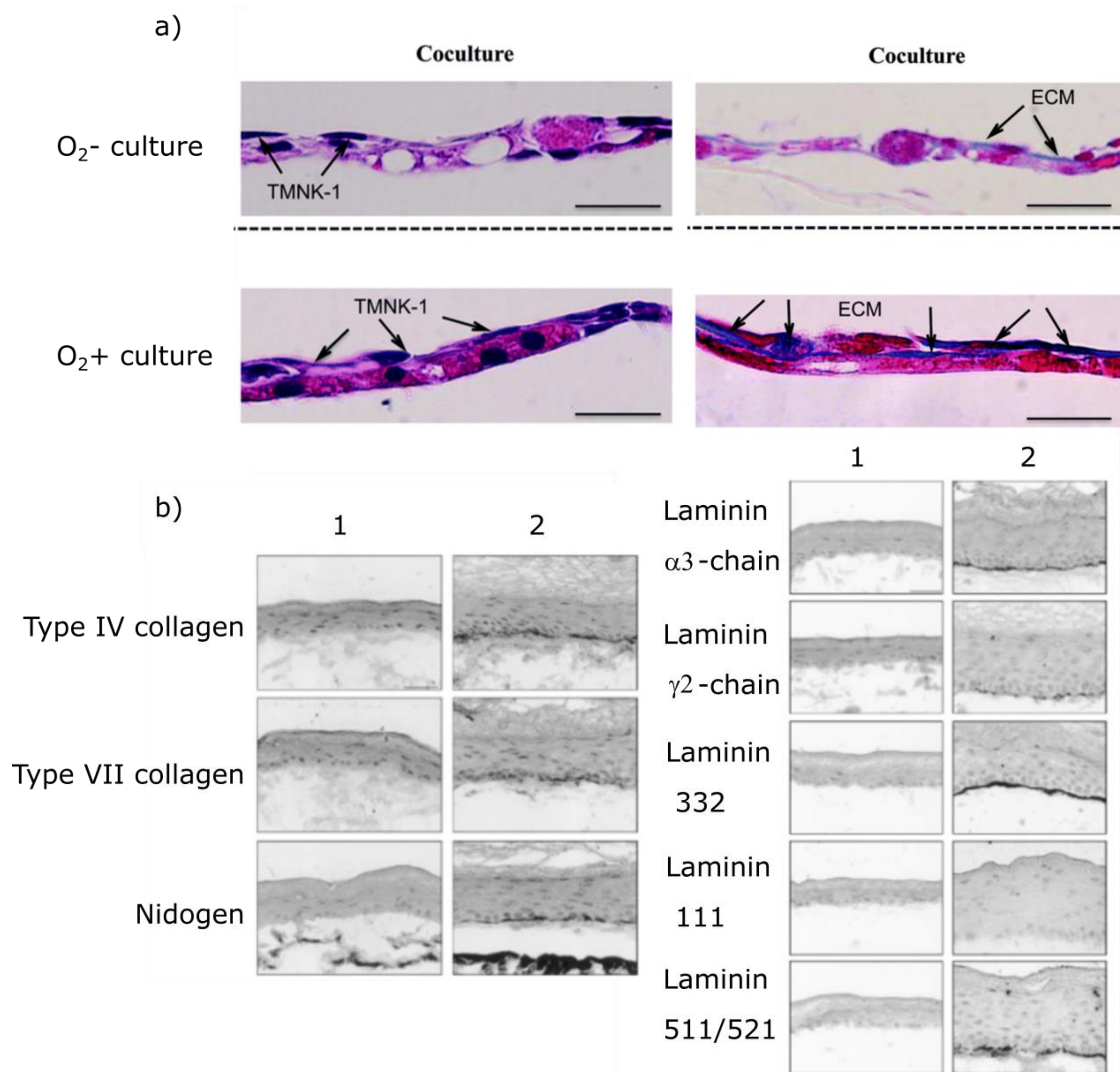


Figure 5 : Characterisation of the coculture effect on cell phenotype and viability. a) Viability and ECM staining of primary rat hepatocytes cultured with liver endothelial cells (TMNK-1) in low oxygenated (O_2- culture) and well-oxygenated culture system (O_2+ culture). Adapted from Ref. 23 with permission from the Royal Society of Chemistry. b) Immunohistochemistry images of the BM components present in keratinocyte culture onto fibroblast-free collagen matrices (1) and onto matrices containing 1 x 10^5 fibroblasts/mL collagen (2). Adapted from Ref. 136 with permission from Elsevier. The original figure[136] was published in Journal of Investigative Dermatology, Vol. 124, Abdoelwaheb El Ghalbzouri, Marcel F. Honkman, Remco Dijkman, Maria Ponec, Basement

Membrane Reconstriction in Human Skin Equivalents is Regulated by Fibroblasts and/or Exogenously Activated Keratinocytes, 79-86, Copyright the Society for Investigative Dermatology, 2005.

The layer-by-layer coculture reproduces the BM biochemical properties in a physiologically relevant manner as it helps to better maintain the cellular phenotype than a simple overlay of ECM gel due to the secretion of not only BM components but also signalling molecules by the cells. To improve this technique, several research groups have included a layer of ECM protein gel[140,141] or used polyelectrolyte layers (chitosan/hyaluronic acid).[142–144] For example, El Ghalbzouri *et al.* demonstrated that fibroblast/keratinocyte coculture enhances the secretion of BM components compared to single culture of keratinocytes as illustrated in **Figure 5b** (1: keratinocytes culture, 2: coculture)**.**[136] The expression of various BM components clearly shows the importance of cross talk between cells for BM formation. However, the addition of this layer can change the BM biophysical properties and to date there is no proof of BM remodelling. The layer-by-layer coculture has two major advantages: (i) its compatibility with bioprinting, which increases the complexity of the culture system by generating a specific pattern with various cell types[145–148] and (ii) the possibility to use cell sheet engineering to generate a complex sheet of cells thanks to a thermo-sensible polymer.[149,150] This cell sheet method can be used to integrate the cocultured cells in an advanced cell-based assay. Nevertheless, its integration within a microfluidic device can be quite challenging in order to keep the structure of the cell layer and its sterility.

IV. Applications of *in vitro* basement membranes

As we described in the section II, *in vivo* BMs are generated by a complex self-assembly of various biomolecules, creating a dynamic 3-D matrix. Due to the difficulty in mimicking this dynamic *in vivo* behaviour in *in vitro* systems, the current models only simulate some of the specific functions of the BM. The limitations of these *in vitro* BMs are mainly due to material issues.[10] In order to perform a given function related to the BM within an advanced coculture cell-based assay, a trade-off has to be made between the material needed for the function and the ease of integrating the material into the required system. This trade-off will be discussed in this section, particular for coculture cell-based assays applied to drug screening and disease modelling. **Table 2** summarizes the capacity of each of the materials (described in the previous section) in terms of properties, integration and applications.

Methods/ Materials	**Biophysical properties**		
	Topography	**Stiffness**	**Cell phenotype**
Polymer membrane	××	×	✓ (due to the ECM
Electrospun scaffold	✓	×	✓ (due to the functi
Peptide-Polysaccharide matrices	×	×	✓
Extracellular matrix protein gel	✓✓	✓	✓
Layer-by-layer coculture	✓✓	✓✓	✓✓

Biochemical properties		Engineering		Applications	
	Degradation/ remodelling	**Integration within microfluidic devices**	**BM component functions**	**Permeability assay for drug screening**	**Disease modelling**
	××	✓✓	×	✓✓	××
	×	✓✓	✓	✓	×
	✓	✓	✓	✓	×
	✓	×	✓	✓	✓
	✓✓	××	××	××	✓✓

Table 2: Capability of the different *in vitro* methods to reproduce *in vivo* BM features (biophysical and biochemical), to be engineered for complex functions and used for drug screening and disease modelling (✓✓: very convenient, ✓: convenient ×: limited, ××: very limited)

a) Drug screening

The development of a new drug requires not only validation of drug efficiency but also measurements of its pharmacokinetic and pharmacodynamics properties including entry and elimination from target cells. Coculture cell-based assays for drug screening should enable the permeability of the BM-mimicking material and cells to biomolecules to be quantified as well as cell viability. A simple model with a BM extract-coated porous polymer membrane can be sufficient to reproduce the BM within permeability assays. Li *et al.* studied the permeability of their coculture system to albumin.[97] They demonstrated that without cells the uncoated membrane is more permeable than a collagen-coated membrane and that GEnCs seems to provide the largest contribution to albumin retention. This is probably due to the fenestrae and glycocalyx formed by the endothelial cell.[151] They also investigated the effect of drugs targeting the podocytes or the GEnCs on albumin permeability. However, their system was restricted to a static analysis, as it did not integrate a microfluidic circuit. To improve the capability of these assays, two different research groups, one in the USA and the other in China developed microfluidic devices based on porous polymer membrane-mimicking BM.[26,100] Musah *et al.* demonstrated that their iPSC-derived podocytes cocultured with GEnCs enhanced the albumin retention compared to a culture of GEnCs only.[100] This result contradicts the findings in the static culture discussed above. The reason for this difference might be due to differences in the distribution of type IV collagen, which is mainly generated by GEnCs in static conditions and by podocytes when mechanical strain is applied.[100]

Zhou *et al.* cultured rodent GEnCs and podocytes within a microfluidic device. They confirmed that the coculture improves the permeability resistance as assessed using labelled inulin, bovine serum albumin and IgG. Their data showed that the contribution of GEnCs to the permeability resistance is more important than of podocytes but in this

system, there is no information concerning the BM components secreted by the cultured cells.

An explanation of these discrepancies in the literature might be due the stiffness of the polymer membrane mimicking the BM and show the importance of the choice of material. For drug screening, in particular permeability assays, the model requires the generation of two different compartments separated by the BM-mimicking material. This separation depends on a material that can be easily handled and has a high mechanical strength. For these reasons, porous polymer membranes are widely employed in permeability assays. Although yet to be used, electrospun scaffolds might be a good candidate for this type of assay due to their topography and the possibility of engineering their biochemical properties. Although their integration was quite limited, the best candidate is probably an ECM protein gel-based membrane due to their biochemical properties and composition.

b) Disease modelling

Various disease processes result in the modification of BM biophysical and/or biochemical properties. These modifications can be due to BM thickening, the modification of the BM microenvironment (*e.g.* hypertension, diabetes), BM invasion by cancer cells or the mutation of a BM component gene (see **table 1**). All these parameters make *in vitro* disease models strongly dependent not only on the materials used to mimic the BM but also on the coculture cell-based assay design. Diseases that are due to changes in the BM microenvironment are the easier to model, particularly in coculture cell-based assays integrating microfluidics. Zhou *et al.* developed a device to mimic hypertensive nephropathy, assuming that higher blood pressure induces higher blood flow rate.[26] They demonstrated that high flow rate (15μL/min) increases the protein permeability and

damages the cell phenotype of both podocytes and GEnCs. Although these interesting results were confirmed by *in vivo* measurements, their device is limited by the non-physiologically relevant properties of their BM-mimicking polycarbonate membrane. Indeed, polycarbonate displays a Young's modulus of 2.5GPa[152], which is three order of magnitude higher than that seen *in vivo*. Furthermore, they did not calculate the value of the shear stress applied to the cells. The BM extract used to coat the polycarbonate membrane, is from EHS tumour, which can contain irrelevant biochemical cues.

To study cancer progression from endothelial-mesenchymal transition (EMT) to the cancer cell adhesion, invasion and vascularisation, various *in vitro* cancer models have been developed. [77,78,140,153,154] Nevertheless, the generation of a good advanced cell-based assay to model cancer still remains challenging as it depends on both biophysical and biochemical properties of BMs. Kamm's group at the Massachusetts Institute of Technology, has been developing advanced cell-based assays to study cancer progression. They demonstrated that cancer progression is affected by various biophysical and biochemical cues.[155–158] However, the relevance of their device is limited because of the use of type I collagen to reproduce the BM instead of type IV collagen and laminin.

To study the effect of BM components, Walter *et al.* demonstrated using a type IV collagen-coated polyacrylamide gel that physical defects within the type IV collagen network triggers EMT, causes MMP-9 inhibition and changes BM stiffness due to the lack of type IV collagen degradation suggesting the beginning of a fibrosis like behaviour.[159] They also generated a type IV collagen-coated type I collagen gel and showed that cells were able to invade the type I collagen gel after undergoing through EMT due to the defects within the type IV collagen network. This work shows the importance of the BM biophysical properties in mechanotransduction/mechanoactivation for cancer research. The authors

acknowledge the limitations of their model due to the lack of BM biochemical cues, especially those from the laminin network. However, they showed the importance of MMP inhibition/activation in such cancer models.

The major role of the proteinases suggests that models using polymer membranes or electrospun scaffolds are not a good choice, as they generally cannot be degraded by proteinases. Furthermore, another important consequence of the proteinase activity is the generation of peptidic fragments called matricryptins, which can exhibit various bioactivities either physiological or pathological.[160–163] To study these effects, the use of only ECM components is required to mimic the BM behaviour. For example, Horejs *et al.* developed a electrospun scaffold containing a fragment of the laminin β1-chain.[164] They demonstrated the potential of this fragment to modulate MMP expression and activity. In using only BM components, Mondrinos *et al.* integrate a performed spheroid of human lung adenocarcinoma cells within their microfluidic device. Although they demonstrated the spheroid adhesion on their membrane, they did not investigate BM invasion and show that their type I collagen membrane is resistant to cell-mediated proteolytic degradation.[27]

Commercially available ECM components from BM extracts have their own limitations because they are specific isoforms of BM components. They can have different biochemical activities due to the generation of different fragments and the remodelling also depends on the surface properties of the material, which support the ECM components.[165,166] For this reason, the rational for the used ECM components mainly depends on the targeted objectives. In the case of *in vitro* model to mimic diseases due to BM component mutation, its generation is still very difficult because of the limited

availability of these mutated components. The development of iPSC technology using cells from patients in order to generate specific BM components is a promising new method to model disease within cell-based assays.

V. Conclusions and future prospects

In vivo basement membranes are complex biological structures, which perform~~s~~ various functions by virtue of their specific components. Furthermore, they display a dynamic behaviour, which is not yet fully understood. For this reason, all the BM functions are not only difficult to recapitulate from both a biophysical and biochemical perspective, but are challenging to integrate in cell-based assays. These assays are generally limited by the low functionalities of the BM-mimicking materials that are used. Currently, the best BM mimics seem to be the functionalized electrospun scaffold and the extracellular matrix membrane, such as those introduced by Rossi *et al.*[24] and Mondrinos *et al.*[27], respectively. These both reproduce the biophysical properties of *in vivo* BMs, except the nonlinear mechanical properties. On the electrospun scaffold developed by Rossi and co-workers[24], the biochemical properties could be tuned by changing the nature of the peptide used. Furthermore, the relative robustness of the scaffolds make them easier to integrate within microfluidic assays. In contrast, extracellular matrix membranes are more difficult to integrate into devices due to their fragile nature, but their offer advantageous in applications such as BM invasion studies.

Future improvements of engineered BMs for coculture cell-based assays will mainly depend on the targeted applications. Applications, in which BM composition is important, such as genetic disorders of BM, will require the development of recombinant BM components and/or the generation of differentiated relevant cells from patient-derived

iPSCs. Applications related to the blood pressure will need a BM-mimicking material, which exhibits nonlinear mechanical properties. For this purpose, the integration of hydrogels, which display stress-stiffening effect[167,168], can be a good solution. However, their biochemical properties will have to be improved by integrating specific BM peptides, for example. Further models integrating both biophysical and biochemical BM properties will not only help to study the contributions of each BM components and their correlation but also to get a better understanding of the drug diffusion through BMs and BM-related diseases. This will enable the development of new drugs and/or the identification of new biomarkers.

Conflicts of interest

There are no conflicts of interest to declare.

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