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Review article

Replication of natural surface topographies to generate advanced cell culture substrates

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ABSTRACT

Surface topographies of cell culture substrates can be used to generate *in vitro* cell culture environments similar to the *in vivo* cell niches. *In vivo,* the physical properties of the extracellular matrix (ECM), such as its topography, provide physical cues that play an important role in modulating cell function. Mimicking these properties remains a challenge to provide *in vitro* realistic environments for cells. Artificially generated substrates' topographies were used extensively to explore this important surface cue. More recently, the replication of natural surface topographies has been enabling to exploration of characteristics such as hierarchy and size scales relevant for cells as advanced biomimetic substrates. These substrates offer more realistic and mimetic environments regarding the topographies found *in vivo*. This review will highlight the use of natural surface topographies as a template to generate substrates for *in-vitro* cell culture. This review starts with an analysis of the main cell functions that can be regulated by the substrate's surface topography through cell-substrate interactions. Then, we will discuss research works wherein substrates for cell biology decorated with natural surface topographies were used and investigated regarding their influence on cellular performance. At the end of this review, we will highlight the advantages and challenges of the use of natural surface topographies as a template for the generation of advanced substrates for cell culture.

1. Introduction

Extracellular matrix (ECM) is a non-cellular and three-dimensional framework present in all tissues/organs [1]. ECM is the substrate responsible for the physical, mechanical, and biochemical support of the cellular components and its interaction with the cells is essential for tissue growth and homeostasis (Fig. 1) [2]. The physical properties of the ECM such as its rigidity, density, porosity, insolubility, and architecture, provide physical cues that play an important role in cell function. The architecture of the ECM is well adapted to each tissue, presenting a very detailed structure [3]. The ECM has a hierarchical structure, with features developing at different length scales (ranging typically from the nanometer to the micrometer). The ECM architecture is uniquely associated to the respective tissue and play a fundamental role in the biochemical pathways responsible for the function of the tissue. The intestinal epithelium is a remarkable example of a tissue that presents a hierarchical structure essential to provide this tissue with specific functionality for nutrient absorption. The basement membrane of the small intestinal epithelium presents a sophisticated invaginated structure with features at various length scales [4]. Those sophisticated invaginated structures form folds that occur at the millimeter scale to increase the overall surface area At the micrometer scale, crypts and villi structures can be observed (tens to hundreds of micrometers in scale), and pores (1–5 μm in diameter) on the surface of the intestinal basement membrane. At the nanometer scale, it is possible to find an interconnected pattern resulting from a fibrous material that provides the ultrastructure of the ECM [4,5]. The ECM hierarchical architecture that is specific from each tissue has raised curiosity to understand their

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influence on cellular behavior. Parameters such as shape, size, topography, and orientation have been studied to investigate the role of ECM architecture on cellular behavior.

Since the beginning of the 20th century, cell biologists have attempted to understand different cellular functions. Most of the studies involve the isolation of cells from living tissues and its *in vitro* subculturing to assess the cellular mechanisms in response to specific stimuli or inhibitions. The major limitation of those studies is using monolayer cell cultures that are typically 2D and do not replicate accurately the *in vivo* conditions [6]. Therefore, it is necessary to improve cell culture systems to become more realistic models of tissue architecture and organization by providing their native physical, mechanical, and biochemical features [7]. To address these properties in cell culture systems it is required to integrate principles of cell biology with materials science [8]. Material science allows for the development of cell culture substrates whose both physical and chemical properties can be modified [9]. Surface topography, wettability, porosity, degradation, or stiffness are examples of physical properties that have been studied in this context [10]. Usually, those modifications aim to generate cell culture substrates that provide a stimulus/support to the cells similar to the stimulus/support provided by ECM in living tissues [11,12]. The ability to generate *in vitro* functional tissues and organs is a challenge that inspires scientists in tissue engineering and regenerative medicine field. Those scientists strive to design solutions to recreate the most critical aspects of living tissues aiming to reconstruct or repair damaged or lost tissues/organs of living organisms [13].

Nature has been used as an inspiration to develop many technologies. For instance, Velcro was one of the first inventions in 1955 embodying the concept of biomimetics once it is inspired by the hooked seeds of the burdock plant that, at the time, leads to the creation of a novel type of zip fastener [14]. To explore the effect of different architectures on cellular behavior, many strategies have been used. Herein, we would like to distinguish two different approaches: the use of artificially generated topographies that can be or not bioinspired and the use of biomimicked topographies. Although some of the artificially generated topographies use natural architectures as an inspiration, this type of topographies does not provide the same amount of the architectural features that are found in nature. Since in the literature it is possible to find several reviews that emphasize the use of artificially generated topographies to explore their functionality in terms of cell performance, this review was focused on the use of natural surface topographies as templates to generate advanced substrates for cell biology. Biomimicking natural surface topographies allows for obtaining topographies similar to the ones found in nature, with a wide range of topographical cues and hierarchical structures.

This review will highlight the use of natural surface topographies as a template to generate advanced substrates for cell biology. In that way, we will by a brief introduction to the main cell functions that can be regulated by the substrate's surface topography through cell-substrate interactions. Then we will discuss research works wherein substrates for cell biology decorated with natural surface topographies were used and investigated regarding their influence on cellular performance. From the analysis of these research works, we present an overview of the most common strategies used to replicate natural surface topographies on biomaterials. At the end of this review, we will highlight the advantages and challenges of the use of natural surface topographies as a template for the generation of substrates for cell biology with its topography and conclude with our future perspectives for the development of this field.

2. Influence of substrates' surface topography on cell performance

The influence of the substrate's surface topography on cell performance has been extensively investigated. In this section, we describe briefly the main cellular functions that have been associated to be influenced by the substrate's surface topography. Fig. 2 presents a schematic representation of the cell performance that is tuned by the interaction between cells and the substrates surface topography. This section presents a brief description of the influence of substrate topography on cell adhesion, cell morphology, cell proliferation, and cell differentiation.

2.1. Cell adhesion and morphology

The intercellular adhesion or the interaction between cells and ECM is crucial for the regulation of several cellular functions resulting from

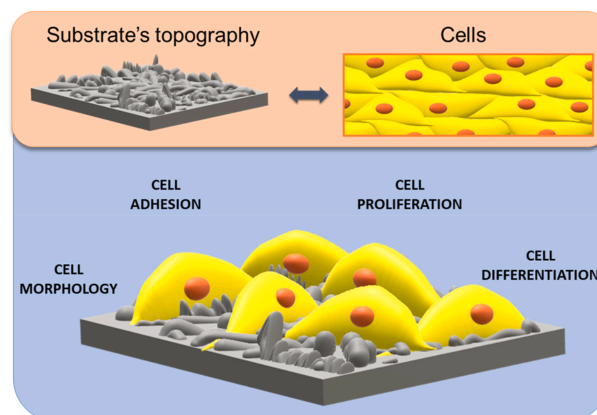

Fig. 2. Cell performance affected by the interaction between cells and the surface topography of substrates.

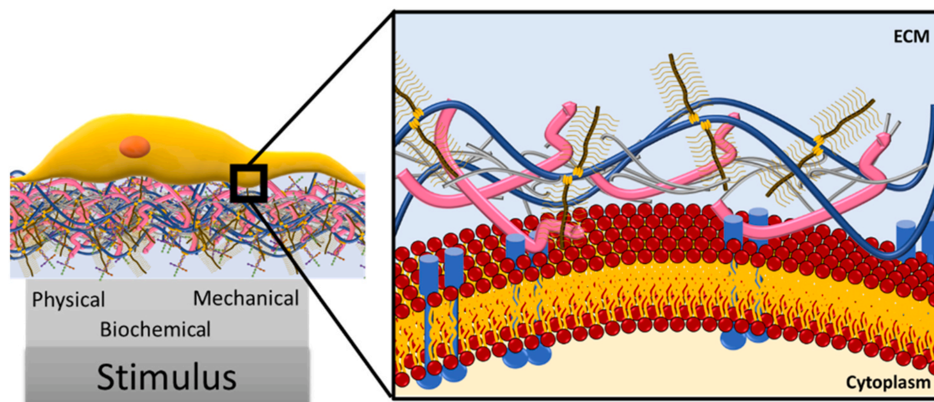

Fig. 1. The stimulus provided by the ECM to tune cell performance.

the stimulation of different signaling pathways such as cell migration, cell proliferation, or differentiation [15]. Moreover, cell adhesion plays an important role in the host integration of implantable biomedical devices [16]. Poor cell adhesion can lead to cell apoptosis or the lack of cell incorporation in tissue engineering approaches that can determine its success [17]. Cell adhesion is dependent on the combination of different surface properties such as topography, chemistry, wettability, and mechanical properties of the substrate (ECM, or biomaterials) [18]. Besides the substrate surface properties, cell adhesion is dependent on proteins that are present in both the cell membrane (with domains on the external side) and the cytoskeleton [19]. On the surface of the cell membrane, integrins are the most associated proteins regarding cell adhesion. Integrins are a family of proteins with a large variety of structures that are associated with several ligand-binding possibilities [20]. This family of proteins presents extra-cellular domains responsible for the interaction with ECM and intracellular domains responsible for the interaction with the cytoskeleton and molecules that regulate different signaling pathways [21,22]. The sites of adhesion of cells to the substrate are designated by focal adhesions. To form these focal adhesions, it is essential to have an interplay between the intracellular component of integrins and cytoskeleton proteins such as actin or tubulin that can be modulated by proteins like talin, paxillin, or vinculin [23]. All these proteins interact with other proteins such as proteases, protein kinases, or signaling molecules that are involved in signaling transduction.

Different aspects of the surface topography have been associated with the influence over cell adhesion. The scale of topographical features is reported to be one of the most important characteristics of the surface of cell culture substrates in the promotion of cell adhesion. Substrates with topographical features that present a scale lower than the cell size (<50 μm) do favor cell adhesion and spreading [24]. With the use of artificially generated substrate topographies containing motifs such as pillars, pits, and fibers with different length scales and organizations, several studies have been made to understand the impact of different parameters on cell adhesion. This topic was extensively revised elsewhere [16].

The way how cells adhere to the substrate can also have implications on cell morphology. Depending on the substrate topography, cells can present different morphologies, tuned by the focal adhesion orientation, distribution, and arrangement. This arrangement determines the direction of the forces exerted by the cytoskeleton and the cytoskeletal tension, which in turn leads to changes in cell morphology, and ultimately cell function [25,26].

2.2. Cell proliferation

Integrins and other cell adhesion molecules regulate gene expression by a signal transduction process. During the adhesion process, the cytoskeleton and particular signaling pathways will be regulated inside the cell. Cytoskeleton proteins such as actin and myosin motors, and several proteins like focal adhesion kinase (FAK) [27,28], src [29], Rho GTPases [29], ERK [30], JNK [30] regulate those signaling pathways and are actively involved in this process.

Compared with flat surfaces, substrates that present topographical cues usually induce an increase in cell proliferation. However, this increase can be dependent on the cell type or dependent on the topographical dimensions, and organization of the substrate. As an example, human umbilical vein endothelial cells proliferate less on nanoscale topography when cultured on a substrate with ridges and grooves ranging from 400 nm to 800 nm when compared to its proliferation on a flat surface [31]. By contrast, similar cells cultured on substrates with a topography that presents grooves ranging from 56 to 61 μm present a higher proliferation when compared to smooth surfaces [32]. Herein we can notice that cell proliferation can be regulated by the substrates' surface topography with an indication that this cell function can be tuned by the scale of the topographical features present on the substrate.

The differences in cell proliferation observed in different substrates with different architectures can be correlated with the proteins involved in the formation of focal adhesions and consequently on cell adhesion. Herein FAK appears to be a central regulator of adhesion-mediated proliferation and it can transduce both stimulatory and inhibitory proliferative signals [29]. Nevertheless, other proteins involved in both cell adhesion and cytoskeleton remodelings such as Rho GTPases, vinculin, tallin, or myosins, are also reported to be involved in the regulation of the cell cycle and consequently in cell proliferation [33].

2.3. Cell differentiation

The topography of biomaterials plays an important role in regulating stem cell fate. The topic of the regulation of stem cells fate by physical cues of the substrates is extensively revised elsewhere [34–36]. The substrate's topography has been reported to be able to regulate the differentiation of stem cells in different lineages. Notable studies report on the induction of differentiation of stem cells on osteogenic [37–39], chondrogenic [40–42], tenogenic [43–46], and neuronal [47–51] lineages. The stem cells start to form lamellipodia and filopodia selecting the ideal way to adhere to the substrate. During the adhesion process, focal adhesions are established. Through mechanotransduction processes, the formation of focal adhesions can regulate several signaling pathways induced by cytoskeleton remodeling. Many different signaling pathways can be activated which can be directly related to the topography-induced gene expression [52]. FAK [53], ERK/MAPK [48], Rho-ROCK [54,55], or Wnt [56–58] signaling pathways are examples of the cascades that can be regulated by the substrate's topography that are directly related to the cell differentiation. The specific topographic features sensed by focal adhesions lead to cell differentiation due to the activation of those signaling pathways that will result in the induction of the differentiation into a certain lineage. For example, the regulation of FAK signaling pathway reveals to be preponderant in determining stem cell fate by topography-induced differentiation. Depending on the substrates' surface topography, this signaling pathway is involved in the determination of stem cell differentiation into osteogenic, neuronal, or tenogenic lineages [37,59,60]. Both micro and nano topographical cues were revealed to regulate the ERK/MAPK signaling pathway and to regulate stem cell differentiation into both adipogenic and osteogenic lineages [61]. Rho family of small guanosine triphosphatases, which have been demonstrated to be key regulators of the actin cytoskeleton. This signaling pathway was considered preponderant in the regulation of topography-induced osteogenic differentiation [61]. The Wnt signaling pathway modulates osteogenic differentiation [62]. Similar to the Rho pathway, Wnt is also a signaling pathway that is closely related to cytoskeleton function [63]. A study using the C2C12 mesenchymal cell line cultured on titanium substrates with different topographies reveals that the actin cytoskeleton alters the cell capability to activate Wnt canonical signaling according to the topography of the underlying substrate [64]. Furthermore, the hierarchical structure of the substrate is reported to have a key role in the topography-induced differentiation process [65].

3. Replication of natural structures and its impact on cellular performance

The use of natural surfaces has been proposed for many different non-biological applications. A well-known example is shark skin, which surface topography, due to its drag reduction effect, was used as a template to produce swimming suits for high-performance athletes [66]. Besides these non-biological uses of natural surface topographies, recently, the use of natural surface topographies in cell biology became more common. Natural surface topographies provide some characteristics that hardly can be achieved in microfabricated substrates. Herein, we do discuss research works where natural surface topographies were replicated and used to study their influence on cell performance. We will

discuss in detail the replication process of natural surface topographies and the impact of the replicated topographies on cell performance. We subdivide this part by the origin of the natural surface topography by plant surfaces, animal surfaces, tissue surfaces, and *in vitro* biology surface topographies. Then we summarize this information in Table 1 of the present manuscript.

3.1. Plant-derived surfaces

Plant surfaces have been studied due to their interesting and unique physical and surface-related properties, including wettability, self-cleaning, anti-reflective, or super-hydrophobic. These properties have inspired the development of materials that mimic those properties. Moreover, recently, the hierarchical structures of plants have been studied to explore their capability to develop surfaces to understand the cell response to substrate topography. Regarding the use of plant surface topographies to study its influence on cell performance, surface topographies from lotus leaves, rose petals, reed leaves and *rubus fruticosus* leaves have been used.

Rose petals have been studied for their superhydrophobic and highly adhesive surface properties. However, recently, the surface topography of rose petals was studied as a template to reproduce its topography for cell culture substrates. Rose petals present a unique microstructure that relies on compactly arranged micropillae with nanoscaled folds on each micropillae (see Fig. 3A). This surface topography was replicated on Polydimethylsiloxane (PDMS) to evaluate the effect of this surface topography on bovine corneal endothelial cells (CECs). In this study, beyond the effect of the surface topography, it was studied the functionalization of the surface of PDMS with Collagen VI and Hyaluronic acid. The substrate topography was revealed to promote higher cell attachment and metabolic activity of these cells and an increase in Na^{+}/K^{+} ATPase, N-Cadherin, and Collagen IV expression [67]. The surface topography of rose petals was also replicated on Honey silk fibroin scaffolds. The replicas obtained through replica molding on PDMS and then on honey silk fibroin scaffolds by drop cast and lyophilization were used to culture adipose-derived mesenchymal stem cells (ADMSCs). The topography of rose petals seems to promote higher proliferation of these cells, a cytoskeletal rearrangement, an upregulation of structural, *trans*-differentiation, and epithelial transformation genes, and down-regulation of senescence-associated genes when compared with a flat surface of the same material [68]. The surface topography of the rose petal was also replicated on PDMS by replica molding and both positive and negative replicas were imprinted through nanoimprint (hot embossing) on PTEG coverslips. NIH-3T3 fibroblast and PaTu8988t cell line cultured on top of these substrates reveal that the surface topography of rose petals possesses a better capability to promote cell adhesion and spreading than flat surfaces [69]. Negative replicas of the surface of Rose petals, parsley leaf (Fig. 3B), and daisy petals (Fig. 3C) were imprinted on hydroxyapatite substrates through microcasting to determine the biological performance of Human adipose-derived stem cells (ADSCs) on top of these substrates. Differences in ADSCs cytoskeleton arrangement were observed comparing those cells cultured on different substrates studied. Moreover, the hydroxyapatite substrates imprinted with the negative replica of the surface of parsley leaf and daisy petals reveal to promote a higher expression of osteogenic associated genes (Collagen I, Runx2, ALP, and Osteopontin) than a flat surface based on the same material. By contrast, hydroxyapatite substrates imprinted with the negative replica of the surface of rose petals reveal to be responsible for a decrease in the expression of those genes when compared to the other analyzed substrates [70]. Overall, the surface topography of rose petals was revealed to be appropriate to develop cell culture substrates improving cell attachment and proliferation, cytoskeleton remodeling, and the activation/inhibition of signaling pathways that regulate gene expression and consequently cell differentiation. Comparing the surface topography of hydroxyapatite substrates imprinted with rose petals (negative), parsley leaf and daisy petals surface topographies, parsley leaf, and daisy petals topographies promote a higher expression of osteogenic associated genes and the negative replica of rose petals topography promote a decrease in the expression of those genes.

Lotus leaves have been studied for their superhydrophobic and self-cleaning properties. The superhydrophobicity and self-cleaning of the Lotus leaves were found to be a result of the hierarchical surface structure built by randomly oriented small hydrophobic wax tubules on the top of convex cell papillae [71]. The surface topography of the lotus leaf has been extensively studied to develop anti-bacterial surfaces. The leaf surface has two levels of structures a microstructure level consisting of surface lumps and a nanostructure level formed by small hairs (Fig. 3D). However, a few studies have been made to evaluate the effect of the surface topography of these leaves on cellular behavior. Lotus leaf topography was obtained by combining multicomponent thermo-curing and replica molding in a polydimethylsiloxane surface containing bromine. Beyond the surface topography, it was studied with the effect of Heparin-like polymers with different chemical compositions on vascular cells and protein adsorption. Human Umbilical Vein Endothelial Cells (HUVECs) and Human Umbilical Vein Smooth Muscle Cells (HUVSMCs) were cultured on top of substrates with the surface topography of lotus leaf and on top of substrates with a flat surface, and by comparison, it was possible to observe that the surface topography of lotus leaf inhibits the cell adhesion and proliferation. However, the two cell types showed different sensitivities to the surface topography effect. Moreover, the surface topography of the lotus leaf reveals to inhibits the adsorption of vitronectin and fibronectin [72]. The surface topography of lotus leaf replicated on poly(3- hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) by replica molding (on PDMS) followed by solvent casting, was used to evaluate the cell behavior of fibroblasts (L929) and endothelial cells (HUVEC). The surface topography of the lotus leaf showed different degrees of inhibition of proliferation for these two cell lines, where the proliferation of endothelial cells was more inhibited than the proliferation of fibroblasts [73]. In summary, when compared with flat surfaces, the surface topography of the lotus leaf is revealed to be inefficient in the promotion of cell adhesion and proliferation. This can be an advantage to designing biomedical devices whose success depends closely on the lack of cell adhesion on their surface. As an example, in catheters the platelet and red blood cell adhesion should be minimized [74].

Reed leaves surface topography presents a ridge/groove microstructure (Fig. 3E) that was used as a template to produce an anisotropic silk film to evaluate the effect of this surface topography on U87 cells' behavior. The surface topography of the leaves was first replicated on PDMS by replica molding, then coated with silk. Studying the influence of both positive and negative replica of the reed leaves surface topography on U87 cells, Zhang and co-workers observed that these cells presented an elongated morphology when cultured on top of the silk films with the surface topography of the leaves when compared with a flat silk film surface. They also verified a distinct orientation of the cells when cultured on the positive or negative replica of the leave surface [75].

The *Xanthosoma sagittifolium* leaf presents a hierarchical surface topography comprising mastoids at the microscale and wrinkles at the nanoscale (Fig. 3F). This surface was replicated on PDMS by replica molding and then the PDMS surface was replicated on poly(*ortho*-methoxyaniline) (POMA) by solvent casting. Culturing rat neural stem cells (rNSCs) on top of this surface and a POMA flat surface, it was not verified significant differences in the adhesion of rNSCs on the studied surfaces. However, after 19 days of culture, it was possible to verify a significant increase of neuronal differentiation of these cells cultured on top of POMA with the surface topography of *Xanthosoma sagittifolium* leaf when compared with these rNSCs cultured on top of POMA flat surfaces [76].

The surface topography of *Rubus fruticosus* leaves was also studied by us intending to understand its potential to improve cell functions. These

Table 1
An overview of the whole biological templates replicated to develop cell culture substrates.

Template	Main Topographical features	Replication method	Materials	Cell type	Effect on cellular performance	Ref.
Rose petals	Microstructure -micropillae Nanoscale- folds on each micropillae.	• replica molding	• PDMS	CECs	• higher cell attachment • higher metabolic activity • an increase in its expression of Na^+/K^+ ATPase, N-Cadherin, and Collagen IV	[67]
		• replica molding • drop cast and lyophilization	• PDMS • Honey silk fibroin scaffolds.•	ADMSCs	• higher proliferation • cytoskeletal rearrangement • upregulation of structural, trans-differentiation, and epithelial trans-formation genes • downregulation of senescence-associated genes	[68]
		• replica molding • nanoimprint (hot embossing)	• PDMS • PTEG coverslips	NIH-3T3 PaTu8988t	• cell adhesion • spreading	[69]
		• microcasting	• hydroxyapatite substrates	ADSCs	• cytoskeleton arrangements • decrease in the expression of (Collagen I, Runx2, ALP, and Osteopontin)	[70]
parsley leaf	Pilar-like microstructures	• microcasting	• hydroxyapatite substrates	ADSCs	• cytoskeleton arrangements • higher expression of (Collagen I, Runx2, ALP, and Osteopontin)	[70]
daisy petals	Honeycomb-like microstructures	• microcasting	• hydroxyapatite substrates	ADSCs	• cytoskeleton arrangements • higher the expression of (Collagen I, Runx2, ALP, and Osteopontin)	[70]
Lotus leaf	• microstructure level-surface lumps • nanostructure level-small hair-like structures	• thermo-curing and replica molding	PDMS surface containing bromine	HUVSMCs HUVECs	• inhibits the cell adhesion and proliferation with different sensitivities to the surface topography effect • lotus leaf reveals to inhibit the adsorption of vitronectin and fibronectin	[72]
		• replica molding • solvent casting	• PDMS • PHBHHx	• L929 • HUVECs	• proliferation of endothelial cells was more inhibited than the proliferation of fibroblasts	[73]
Reed leaves	Ridge/ groove microstructure	• Replica molding • Coating with silk	• PDMS • Silk	U87	• Elongated morphology • Distinct orientation	[75]
Xanthosoma sagittifolium leaf	Microscale- mastoids Nanoscale- wrinkles	• replica molding • solvent casting	• PDMS • POMA	rNSCs	• increase of neuronal differentiation	[76]
Rubus fruticosus	hierarchical size-scale surface topography whose main motifs range from nano to micron scale	• replica molding • NIL	• PDMS • PCL	L929	• Higher Proliferation • Higher metabolic activity	[77]
				Ea.hy926	• heterogeneous spatial distribution	[78]
Sharkskin	microstructures - "denticle"	• replica molding • solvent casting	• PDMS • chitosan	L929 HaCaT	• enhances cell viability • higher available surface for cell adhesion and spreading	[82,83]
Oyster shell	**Nacre** small tablet features	• replica molding	• PDMS	MSCs	• an increase in *OPN*, *OCN*, and *ALP* expression	[84–86]
	Prism larger polygonal prisms	• NIL	• PCL		• any indication of mature development • increase in *CD63* expression and retaining STRO-1 expression • retains the stem cell multipotency and plasticity *in vitro*	
Small Intestine epithelium	macroscopic folds, villi approximately 50–150 µm wide and 100–200 µm tall, crypts 20–50 µm in diameter, 1–5 µm pores, and extracellular matrix (ECM) fibers, such as collagen, that are approximately 50 nm in diameter.	• chemical vapor deposition • replica molding	• parylene • PDMS	Caco-2	• Changes in morphology • increased ALP activity indicator of the Caco-2 differentiation	[5]
Tendon	hierarchical arrangement of parallel collagen fibrils and fibers that adopts a crimp-type/wavy configuration	cryosections		MC3T3E1, 3T3,htMSC, MDCK, HeLa	•different morphology •higher expression of tenomodulin	[87]
		replica molding • NIL	PDMS polystyrene	Tenocytes isolated from rat Achilles tendon	•similar morphology to in vivo tenocytes •lower rate of proliferation • phenotype similar to tenocytes *in vivo*	[88]

PDMS= Polydimethylsiloxane; CECs = bovine corneal endothelial cells; ADMSCs = adipose-derived mesenchymal stem cells; ADSCs= Human adipose-derived stem cells; Runx2 = Runt-related transcription factor 2; ALP= Alkaline phosphatase; HUVSMCs= Human Umbilical Vein Smooth Muscle Cells; HUVECs= Human umbilical vein endothelial cells; PHBHHx = poly(3- hydroxybutyrate-*co*-3-hydroxyhexanoate); POMA = poly(*ortho*-methoxyaniline); rNSCs = rat neural stem cells; NIL = nanoimprint lithography; PCL = polycaprolactone; OPN= Osteopontin; OCN= Osteocalcin; htMSC= Human Turbinate Mesenchymal Stromal Cell; MDCK = Madin-Darby canine kidney cells.

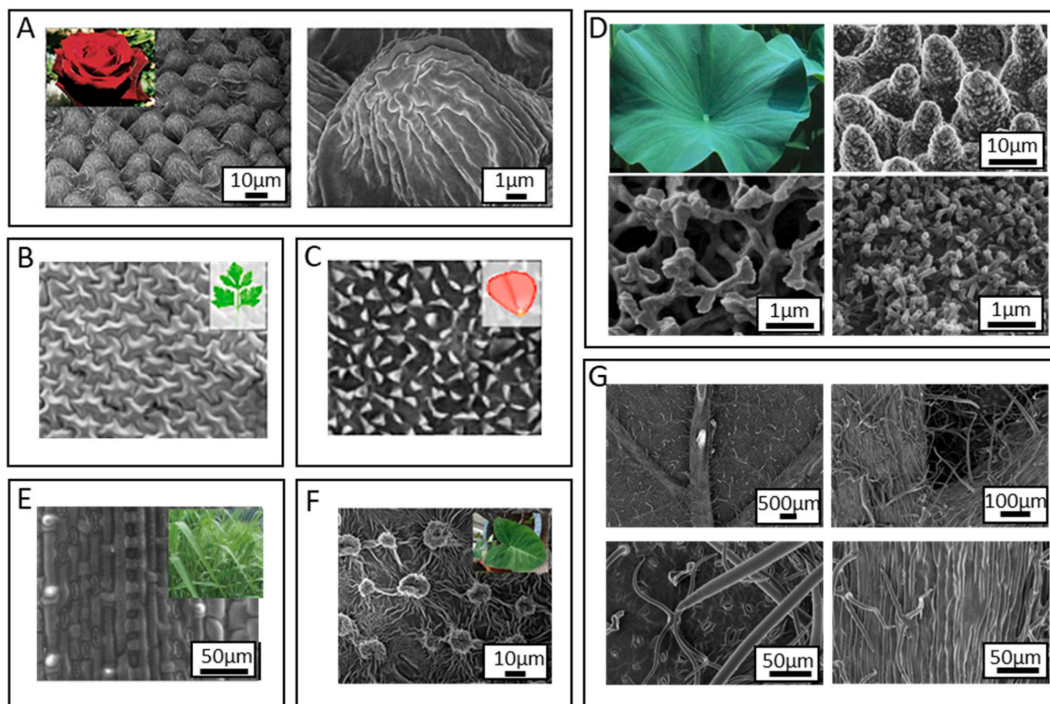

Fig. 3. Hierarchical structures presented on the surface of plants that were used to be replicated to develop cell culture substrates. A- Rose petals (adapted from Ref. [79]); B- Parsley leaf (adapted from Ref. [70]); C- Daisy petals, (adapted from Ref. [70]); D- Lotus leaf (adapted from Ref. [80]); E– Reed leaves (adapted from Ref. [75]) F- *Xanthosoma sagittifolium* leaf (adapted from Ref. [76]); G- *Rubus fruticosus* leaves [77,81].

leaves present a hierarchical size-scale surface topography whose main motifs range from nano to micron scale. Many topographical structures can be found on its surfaces such as the veins and the stomate structures. The veins present a fibrillar-like structure with aligned fiber-like structures with 5343 ± 1605 μm of diameter and the stomate presents a less prominent topographical structure. It is also possible to observe that each fiber-like structure presents smaller topographic features (Fig. 3G). This surface topography was firstly replicated on PDMS by replica molding and then imprinted on PCL spin-casting membranes by nanoimprint lithography (hot embossing). The studied surface topography reveals to be more prone to support L929 cells proliferation also enhancing its metabolic activity [77]. Moreover, the angiogenic potential of this surface topography was also tested. Culturing Ea.hy926 cells on top of both flat and biomimetic patterned PCL membranes it was possible to observe that biomimetic patterned PCL membranes are more prone to support those cells proliferation. Furthermore, on top of biomimetic patterned PCL membranes, Ea.hy926 cells reveal present a heterogeneous spatial distribution after 7 days of culture, being more present on the region of the PCL membranes that contains the biomimetic leaf's veins topographical features. Additionally, it was verified an improved angiogenic capacity by the Rubus fruticosus leaves surface topography when compared with a flat surface on a chick chorioallantoic membrane assay, an *in ovo* methodology to evaluate the angiogenic potential of biomaterials [78].

3.2. Animal-derived surfaces

Animal surfaces present unique structures and physical properties that can be useful to mimic several biological human functions. The most known example is the gecko feet. The gecko feet present a unique structure that confers to this animal the ability to move on vertical surfaces or in ceilings due to its strong adhesive properties that are reusable and easy to detach [89]. Gecko feet inspired the development of tissue adhesives for sealing wounds [90]. Other animal surfaces such as sharkskin and oyster shells were studied to understand their impact on cell performance. Moreover, some tissues that present a remarkable surface topography such as tendons and the small intestine surface have been replicated on biomaterials to assess their impact on cell performance.

Sharks have attracted the attention of researchers for the past few decades due to two main characteristics of their skin: drag-reduction and antifouling properties. It is speculated that sharkskin remains free of microorganism adhesion probably due to the riblet-like structure called denticles (Fig. 4A). These unique microstructures on the sharkskin surface known as "denticle" decreases the friction forces at the interface of water and skin leading to reduced drag force and increased swimming speed of the shark. It has also been shown that these denticles prevent bacterial biofilm formation both in static and dynamic conditions. Intending to study the effect of the sharkskin surface topography on biofilm formation and cell performance, sharkskin was replicated on chitosan membranes via replica molding (to produce negative replicas of the sharkskin surface topography on PDMS) and solvent casting. Besides the effect of the reduction of biofilm formation, it was observed the culture of L929 and HaCaT cells on top of the generated replicas of sharkskin surface topography enhance cell viability and provide a more available surface for cell adhesion and spreading [82,83].

The oyster shell (*Pinctada maxima*) has been studied in the field of bone tissue engineering since it resembles bone composition due to the inorganic, mineralized matrix and an organic fraction composed of proteins. The oyster shell presents two different kinds of surface topography on the nacre and the prism side. The surface of the nacre is composed of small tablet features and the prism side is composed of larger polygonal prisms (Fig. 4B). Envisioning to evaluate the effect of both surface topographies on stem cell behavior, PDMS-negative replicas of both surfaces were produced by replica molding. Then, by nanoimprint lithography (hot embossing), both surfaces were imprinted on PCL. Human mesenchymal stem cells (MSCs) on top of both surface topographies reveal present different behaviors. On the nacre replica, it was verified the induction of osteogenic differentiation of those cells, with an increase in OPN, OCN, and ALP expression, compared to flat

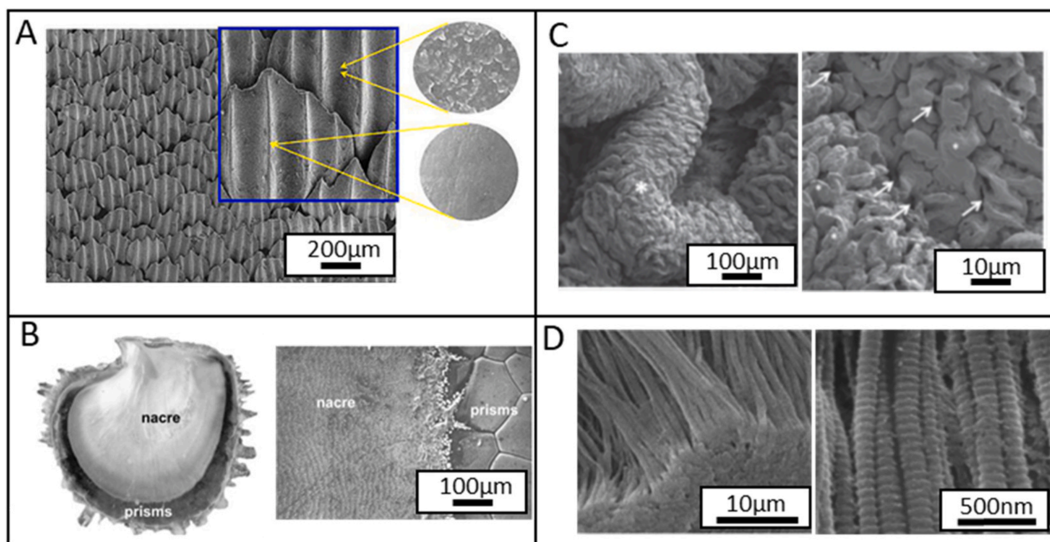

Fig. 4. Hierarchical structures presented on the surface of animal tissues that were used to be replicated to develop cell culture substrates. A- Sharkskin (adapted from Ref. [91]); B- Oyster shell (adapted from Ref. [92]); C- Small intestine, (adapted from Ref. [5]); D- Tendon (adapted from Ref. [93]).

control PCL surfaces. On the prism replica, those cells did not show any indication of mature development, along any lineage exhibiting an increase in CD63 expression and retaining STRO-1 expression. Moreover, those cells cultured on prism replica maintain the differentiation potential to differentiate into the osteogenic and adipogenic lineage. Those results suggest that the surface topography of the prism of the oyster shell retains stem cell multipotency and plasticity *in vitro* [84–86].

The epithelium of the small intestine is composed of a tight monolayer in contact with the underlying basement membrane. The basement membrane is composed of complex, irregular, 3D features over multiple length scales, including macroscopic folds, villi approximately 50–150 μm wide and 100–200 μm tall, crypts 20–50 μm in diameter, 1–5 μm pores, and extracellular matrix (ECM) fibers, such as collagen, that are approximately 50 nm in diameter (Fig. 4C). The replica of the surface topography of the small intestine was obtained replicated by chemical vapor deposition on top of parylene on the tissue and then replicated on PDMS by replica molding. Caco-2 cells were cultured on both PDMS flat surface and PDMS with the surface topography of the small intestine presenting different morphologies. Moreover, after 10 days of growth in standard culture conditions, those cells exhibit a significantly increased ALP activity on biomimetic PDMS growth substrates compared to flat PDMS substrates. This is an indicator of the influence of this topography on Caco-2 differentiation [5].

Tendon is a unique type of connective tissue that transmits muscle contraction forces to bones to produce motion and maintain body posture. In a healthy tendon, a typical hierarchical arrangement of parallel collagen fibrils and fibers forms a tendon unit, which is an unloaded state that adopts a crimp-type/wavy configuration (Fig. 4D). From a structural point of view, the organization of collagen fibers changes from highly anisotropic to more isotropic, and at the micro-level, they become more angulated, and the number of small-diameter collagen fibers is increased. Using tendon cryosections, it was verified that different types of cells (MC3T3E1, 3T3, htMSC, MDCK, and HeLa) present different morphologies when cultured on top of these cryosections. Moreover, besides the morphological aspects, it was verified that MSCs cultured on top of tendon sections presented fewer proliferation rates than when cultured on glass, however, those cells cultured on top of tendon cryosections presented a higher expression of tenomodulin. These tendon cryosections were replicated on PDMS by replica molding and coated with collagen. Those replicas reveal have the potential to promote the tenogenic differentiation of MSCs [87]. The surface topography of the tendon was also replicated on polystyrene via replica molding (on PDMS) and nanoimprint lithography (hot embossing). Tenocytes isolated from rat Achilles tendon cultured on top of these replicas reveal to have a similar morphology to *in vivo* tenocytes. Moreover, when compared with isolated tenocytes cultured on flat polystyrene surfaces, tenocytes cultured on top of the polystyrene with the replica of the tendon surface topography present a lower rate of proliferation, however, its phenotype is more similar to the phenotype of the tenocytes *in vivo* [88].

4. Overview of the strategy to replicate natural surface topographies

Overall, the strategy to develop cell culture substrates mimicking a natural surface topography follows the same basic steps. Firstly, the natural surface is immobilized and depending on its stiffness may need to be fixated chemically (for example with formalin). Then, the next step to replicate a natural surface topography on biomaterials involves the production of a negative replica of the surface, usually through a replica molding process. Replica molding is a technique that allows transferring the selected pattern from one material to another. This technique allows duplicating of structure shapes, sizes, and patterns in a wide range of materials [94]. PDMS is a gold-standard elastomeric polymer to be used in replica molding methodologies. The replication technique consists of three essential steps: 1) Selection/fabrication of the surface structure to be replicated; 2) transferring this surface pattern to PDMS by curing a PDMS prepolymer in contact with the master and releasing the PDMS from the master; 3) peeling off the cured PDMS from the sample [95]. A major advantage of replica molding is that it can create molds that cover a wide range of surfaces including non-planar curved shapes, and large area masters.

When the PDMS mold (negative replica) is obtained, there are several techniques that can be used to obtain a substrate with a positive replica of the natural surface. These techniques depend on the material that was selected being the most common approaches the solvent casting, coating techniques, nanoimprint lithography and hot embossing. A schematic overview of the replication technique is shown in Fig. 5.

5. Advantages and challenges of the use of natural surface topographies

There are several advantages of using natural surface topographies when compared to substrates with artificially generated topographies.

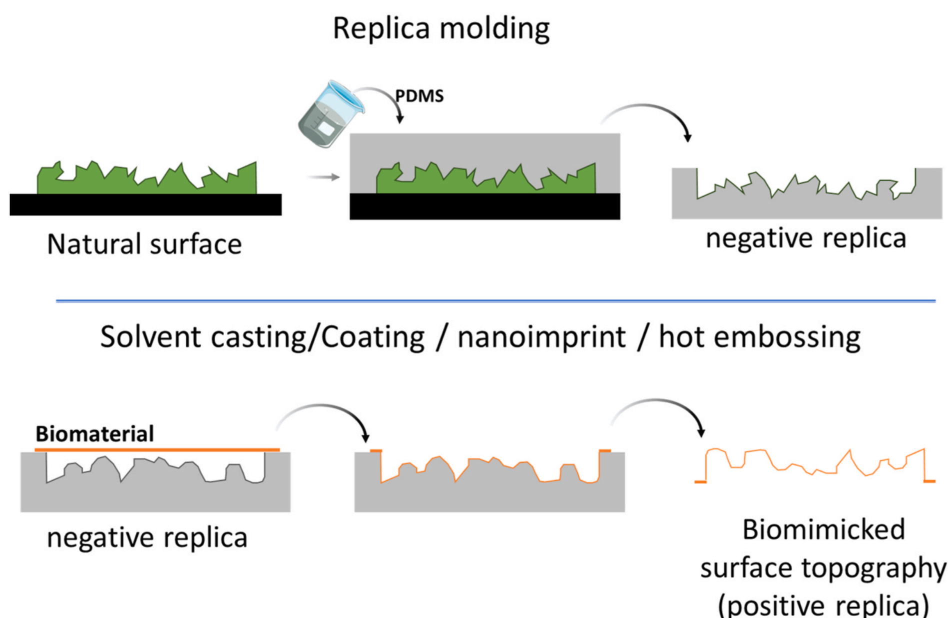

Fig. 5. Main stages of the techniques used to obtain cell culture substrates with natural-derived surface topographies.

Firstly, in nature, we can find an infinity of surface topographies that are suitable to be replicated to generate advanced topographies for cell culture substrates. We can find so many different natural structures and each one has unique features. Moreover, several biological functions have been reported to be influenced by the surface topography of the substrates and different cells present different responses to the same substrates' topography. As such, the combination of different biomimetic surface topographies and different cell types gives rise to an infinity of opportunities to develop further studies. These nature-derived topographies present unique hierarchical architectures and they are hardly achieved in artificially generated topographies. This aspect can provide unique physical properties and functionalities that can be useful in the development of new functional cell culture substrates. Secondly, nature-derived surface topographies present a combination of different topographical features on the same surface whose combination can synergistically work to regulate a certain cellular function. Usually, artificially generated topographies present a limited number of topographical cues that can be insufficient to obtain certain functionalities. We strongly believe that biomimicking the architectures of natural surfaces can give rise to a better understanding of the physical stimulus that is provided to cells. This understanding can give rise to new approaches that can be useful not only to develop advanced cell culture substrates but also in a tissue engineering scenario. Furthermore, the techniques that are commonly used to obtain replicas of these natural surfaces are highly cost-effective, most of the time do not require sophisticated equipment when compared with the production of substrates with artificially generated topographies.

Besides the presented advantages, the use of natural surface topographies as a substrate for cell culture can also be challenging. The standardization of natural surface topographies can become a problem due to the difficulty to circumvent the inter-variability of the substrates, such as the leaves of a particular plant. The variation from batch to batch is intrinsic resulting in small differences regarding the topographical cues. These small differences can also be observed in samples obtained in different stages of development. Moreover, surfaces from nature can be submitted to different environmental conditions that can affect their surface pattern. Considering the leaves, for example, higher temperatures can develop in a plant the necessity to prevent the loss of the water content leading to a shrinkage of their leaves that can change the patterning of its surface [96]. Regarding the use of animal tissues, some concerns considering the regulation of the use of animals for research purposes can compromise its use.

6. Future perspectives

In nature, we can find a large variety of surface topographies that are suitable to be replicated to generate cell culture substrates. As demonstrated above, the use of natural surface topographies can be a very attractive strategy to enhance several cell responses. Despite the challenges identified above, the progress achieved seems quite promising. Thus, new strategies involving the development of powerful screening identification of high-performance biomimetic culture substrates are needed. As such, testing in high throughput systems with many different biomimetic surface topographies and different cell types allows conducting studies at a larger scale in a cost and time-effective way. This approach would create the possibility to find particular traits or motifs about topographical cues and their hierarchical arrangements that are more effective in obtaining high-performance bioactivity. Additionally, long-term studies and the study of molecular mechanisms that are on the basis of that bioactivity should be followed. Moreover, although considerable efforts have been made in studies on the effect of biomimetic surface topographies on cells *in vitro, in vivo* animal experiments using biomimetic topographies are not yet routinely performed. For the successful translation of this approach, a large number of *in vivo* data must be generated in the future. Furthermore, it is also important to access the difference between biomimetic 3D topographical structures and biomimetic 2D topographical structures. In that way, it will be possible to explore in a better manner the potential of the use of biomimetic topographical structures in approaches with strong translational potential. From a long-term perspective, envisioning that some of these topographies will reveal being useful in future clinical applications, it would be necessary to overcome some current challenges presented by the use of biomimetic surface topographies. One of them is standardization. The variation from batch-to-batch samples (inherent to biological templates) resulting from small different individual topographical details can difficult it to standardize. Herein, the production of reliable stamps whose topography can be replicated many times, and whose topography can be used repeatedly to produce new stamps can be a candidate solution to overcome this problem. Finally, a big challenge that the use of natural surfaces faces is the scale-up production. Until now, this approach is only available at the lab scale. Efforts should be devoted in the future to overcome this problem, such as the processing of

samples by injection molding or roll-to-roll lithography.

7. Conclusions

Natural surface topographies are emerging as a biomimetic strategy to produce architectures very difficult to find in artificially generated topographies. Besides its several applications, herein we focus on their impact on cell performance and responses. In conclusion, we observed that replicating topographies directly from nature surfaces provides a unique ability for the development of cell culture substrates with exclusive hierarchical arrangements of topographical features. These architectures revealed providing physical stimuli has specific benefits for cell performance. This approach allows an understanding of some mechanisms that underly the cellular response to the topography of the substrates. Moreover, it can facilitate the development of new therapeutic strategies within the tissue engineering and regenerative medicine fields. Despite several technological challenges still in need of progress, the use of natural surface topographies has strong potential to grow and to be further exploited in Biomedical applications.

Declaration of competing interest

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

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