

Regulation of cell fate by cell imprinting approach in vitro

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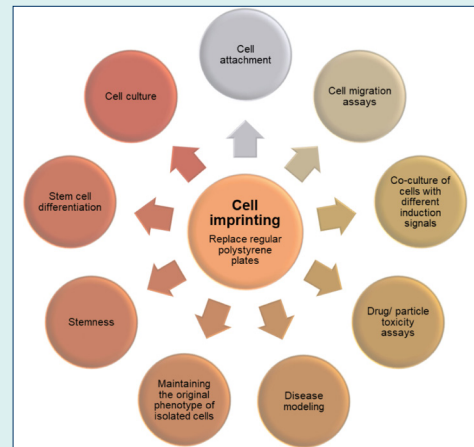
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Abstract

Cell culture-based technologies are widely utilized in various domains such as drug evaluation, toxicity assessment, vaccine and biopharmaceutical development, reproductive technology, and regenerative medicine. It has been demonstrated that pre-adsorption of extracellular matrix (ECM) proteins including collagen, laminin and fibronectin provide more degrees of support for cell adhesion. The purpose of cell imprinting is to imitate the natural topography of cell membranes by gels or polymers to create a reliable environment for the regulation of cell function. The results of recent studies show that cell imprinting is a tool to guide the behavior of cultured cells by controlling their adhesive interactions with surfaces. Therefore, in this review we aim to compare different cell cultures with the imprinting method and discuss different cell imprinting applications in regenerative medicine, personalized medicine, disease modeling, and cell therapy.



Introduction

Tissues are comprised of cells and their local surroundings, including the extracellular matrix (ECM). The ECM consists of different proteins in a three-dimensional (3D) structure with biophysical and biochemical signaling that is specific to each type of cell. These signals play a key role in diverse cell functions including attachment, proliferation, migration, expression and differentiation.^{1,2} For cell-based experiments involved in research or clinics, an artificial ECM is required to support cell function, which can be considered as a substrate. Cells are cultivated in a substrate sense and respond to the physical/chemical properties of the contact surface. The chemical interaction of cultured cells with substrates is gradually affected by the presence of small molecules, ions and proteins in media.

On the other hand, the physical characteristics of a surface are characterized by hydrophobicity, charge, topography and viscoelasticity.^{3,4} The consequences of the substrates' physical/mechanical properties on cell proliferation and differentiation have been extensively researched.^{5,6}

There are different methods employed for 2D or 3D cell cultures in vitro. Monolayer cell cultivation in laboratories commonly relies on conventional polystyrene plates. Nevertheless, it is important to note that the physical and chemical characteristics of these 2D substrates diverge significantly from those found in natural environments. In such a way that cell-cell and cell-extracellular environment communications are not demonstrated in 2D conditions as they would be in tumor aggregates. These interactions manage cell differentiation, proliferation, cellular survival,



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expression of genes and proteins reaction to stimuli, drug metabolism, paracrine effects and other cellular responses. For example, the association of $\beta 1$ -integrin and epidermal growth factor receptor signaling was recognized in 3D culture but not seen in 2D.^{7,8} Therefore, it is proposed that 3D cultures can be utilized to bridge the gap between 2D and in vivo conditions in various areas such as cancer research, drug discovery, neuroscience, and regenerative medicine.

The methods for 3D culture can be classified as either scaffold-based or non-scaffold-based. The scaffold-based methods involve the use of hydrogels, fibers, or sponge-like structures made from natural or synthetic biomaterials. These structures are created using techniques such as bio-printing, freeze-drying, or electrospinning. In 3D non-scaffold-based methods, cells will be cultured in the form of spheroids, organoids, or pellets using low attachment flasks, microfluidic systems or bioreactors.⁸⁻¹⁰

Recently, the cell imprinting method has been suggested as a high throughput and cost-effective strategy for manufacturing pseudo-3D cell culture substrates. In this strategy, settled cells are utilized as formats to replicate negative designs of the cells' membranes. These particular bio-geometrical properties are utilized as physical signals to balance cell reactions such as cell migration, adhesion, proliferation, and differentiation.¹¹ Utilizing cells for cell-imprint makes micro-/nano structures with dynamic areas which can be engaged in within the cell-surface intuitively. Given the significance of improving the cell culture, in this review we intend to discuss the interaction between cells and various physicochemical attributes of culture substrates in 2D, 3D, and cell imprinting.

Cell culture methods in vitro

An in vitro cell culture is a procedure utilized to investigate the behavior of animal cells in a regulated environment that imitates nature without ideally changing cell morphology and function. Presently, there are various types of cell cultures. Animal cell cultures have been used to investigate fundamental cell biology, interactions of drugs or materials with cells, and the generation of vaccines and proteins, etc. Amphibian cells were selected as the primary model for the cell culture because cells in exothermic animals do not require consecutive incubation periods. Subsequently, advances in biology increased focus on endothermic animals, where normal and pathological expansion is the same as that of humans. The availability of genetically pure mouse strains facilitated the investigation of mammals in laboratory settings.¹² Because the culture medium is the most important factor in cell culture technology, researchers must evaluate different cell culture procedures to choose a suitable medium or modify previous ones to suit their purposes. Artificial media can be classified into several groups based on the type of supplemented materials: serum-containing media, serum-free media, protein-free media, and defined

chemical media.¹³

In order to conduct laboratory investigations, researchers frequently employ a variety of procedures in separation, fractionation, and characterization to prepare tissue samples accordingly. The selection of each method depends on the favorable level of separation, maintenance of viability, and technical investigation to be evaluated. Generally, highly efficient cell separation techniques rely on various factors such as cell size, cell density, cell charge, cell surface chemistry, cellular complexity and fluorescence effusion of two or more cellular components or adsorbed antibodies.¹²

In the next step after isolation, the cell cultures can be grown in a suspension form in the medium or allowed to adhere to a glass or plastic dish depending on the nature of the cells and their microenvironment.¹⁴ The most frequently utilized type of cell culture is the two-dimensional (2D) model; however, recently 3D culture procedures have increased in popularity.^{15,16}

Tissue culture plates

In 2D cultures, cells adhere to polystyrene-based surfaces and are expanded as a monolayer in a culture flask or flat Petri dish.¹⁷ The principal advantage of this method is its simplicity in using a group of cloned cells. Cell cultures require significant control of the physicochemical environment (i.e., pH, temperature, osmotic pressure, oxygen, CO₂ tension and chemicals), which must be regulated accurately to obtain reproducible results.¹² While 2D cultures on smooth rigid polystyrene-based culture dishes are the most prevalent types of culture, mammalian tissues are predominantly pliable and textured structures. It is well known that both rigidity and roughness affect cell function in vitro.¹⁸

Therefore, to better mimic the natural environment in vitro, factors such as forced polarity, flattened cell shape, mechanical/biochemical signals, and subsequent cell-to-cell communication must be regulated.^{19,20} Otherwise, the 2D monolayer environment changes the function of the cells and disturbs the final outcome. For example, Proper assessment of significant properties of cancer cells is not achievable in 2D cultures.²¹ Hence, the main disadvantage of the 2D environment is its inability to mimic the physical mechanical properties of the cell ECM. In addition, in the 2D environment, cell-cell and cell-extracellular interactions are not the same as in the tumor environment. These communications can cause cell proliferation, differentiation and gene or protein expressions in response to chemical stimuli such as drugs or small molecules.²²⁻²⁴ Isolation of cells from their natural 3D environment and subsequent cultivation in vitro on 2D substrates changes the original morphology of the nucleus and cytoplasm, and affects cell function in response to signals.^{25,26} As a result, after several cell divisions, the cells lose their polarity during coherent expansion due to the presence of aberrations in the DNA

spatial structure, which leads to the reaction of those cells to apoptosis and other phenomena.^{27,28} A further disadvantage of the 2D culture is that the monolayer cells have inadequate access to the essential components of the medium, including oxygen, nutrients, metabolites and signal molecules.²⁹ Therefore, there are no similarities between the microenvironment of a cell niche and the case of an *in vitro* monolayer culture. In the case of tumors where several types of cells are involved, there are noted alterations in gene expression, splicing, topology, and cell biochemistry in the 2D culture.^{30,31}

These disadvantages of 2D cultures encouraged researchers to find another model that could more aptly mimic the natural environment of cells in tissues including 3D culture systems.³²

Organoid, pellet, spheroid, 3D scaffolds and hydrogels

An appropriate gradient for nutrients and waste is required to enable 3D culture media for supporting cell growth that most closely resembles the *in vivo* environment. The invention of 3D cell culture methods that mimic the *in vivo* interactions of tissues and organs has created new opportunities for researchers to investigate the biochemical and biomechanical signals during cell development.^{21,33} Here, we discuss the various 3D cell culture techniques such as organoids, pellets, spheroids, 3D scaffolds or hydrogels that have been developed to simulate *in vivo* conditions for cell growth.

Organoids are 3D multicellular structures cultivated *in vitro* that replicate the characteristics of an organ. They serve as a valuable tool for studying specific aspects of an organ within a controlled laboratory setting.³⁴ Organoid cultures are potent methods progressively used for various investigations including regenerative medicine, disease modeling, drug discovery and personalized medicine. Organoids can be obtained either from pluripotent stem cells (PSCs) or adult tissue-resident cells, whether stem or differentiated cells and embryonic progenitors.³⁵ An organoid is considered to be a 3D construct developed from stem cells including organ-specific cell types that are being self-organized via cell sorting and spatially restricted lineage commitment.³⁶ Organoid models are more useful than other cellular models, especially in the heterogeneities of cancer, hereditary genetic disorders (such as cystic fibrosis) and host–pathogen interplays.^{37,38} It has been shown that organoids contribute as great tools in studying tumorigenesis and cancer development *in vitro* and represent a great potential for translational studies.^{39,40} Long ago, the approach of tumoroids, a promising elective culture show to the routine 2D system, has been driven to critical advances in understanding complex cancer cell science. The term “tumoroid” implies “tumor-like organoid”: tumoroids regularly evolve from primary tumors gathered from oncological patients.⁴¹ Cancer physiopathology can further be elucidated through specific investigations on some major aspects

of characterizing the tumor microenvironment (TME). Moreover, the pathways included in the initiation of cancer and cell-to-cell connection, as well as the role of TME in cancer development are yet remained unexplained; hence, tumoroids may play a vital role in cancer biology.^{42,43} Upon replacement with the supporting matrix, cells inside the tumoroids can partially, imitate the *in-vivo*-like conditions in which morphology and cellular interactions closely resemble the primary niche. These appearances result in acquiring useful information that mimics to the human body. Tumoroids have been employed to realize if the healthy mammary microenvironment can induce a physiological action in breast cancer cells, and the reaction of cancer cells to new drugs.⁴² To better explore from this viewpoint, tumoroids have been made using breast cancer cells and ECM. These components have the potential to induce enormous differentiation and growth of stem cells derived from other organs after their injection into the mammary glands of mice.⁴⁴ In another study, the tumoricidal properties of melatonin on colon cancer tumoroids in three different cell lines including HT29 adenocarcinoma cells, fetal foreskin HFFF2 fibroblasts and HUVECs (Human umbilical vein endothelial cells) to emulate *in vivo* conditions were evaluated *in vitro*. It was stated that the 3D tumoroid system is a promising approach for the assessment of anti-cancer compounds as compared to the conventional 2D culture system and melatonin was able to significantly decrease the cohesion of tumor mass through the arrest of some angiogenesis agents such as endocan with simultaneous induction of necrotic variations.⁴⁵

However, there is limited use of organoid methods for complex and advanced diseases that are not connected to tissue growth or monogenetic drivers. In such cases, distinct phenotypes such as those in many polygenic autoimmune conditions, may not indicate the originating cell status or relevant environmental signal in an organoid method.⁴⁶ Through a study on liver organoids, Huch et al reported that cells may not reach full maturity, presumably because common culturing procedures do not recreate the body’s complete biochemical and physical environment.⁴⁷ For instance, organoids located outside the body of an animal do not experience natural directional signals that accurately arrange the body’s cells from top to bottom or left to right.⁴⁸ A major drawback is the inability of researchers to create organs that have blood vessels. The organoid’s size and complexity are constrained due to the absence of blood circulation.⁴⁹ Therefore, it is necessary to consider systematic approaches to determine the advantages and disadvantages of the organoid model.

Another model is the pellet culture of the chondrocyte *in vitro*. It is well known that the original phenotype of chondrocytes changes to a fibroblast-like phenotype after isolation from the body and subsequent cultivation *in vitro*.

The pellet culture is a constant, biomaterial-free

culture system that is well-suited for both chondrocyte redifferentiation and mesenchymal stem cell (MSC) differentiation studies *in vitro*. The pellet culture exhibits a significant cellular density, promoting extensive cell-cell contacts.^{50,51} Traditionally, the word pellet has been applied to describe consistently constructed geometrically determined agglomerates created from diverse beginning materials by the use of various processing conditions. Pellets for pharmaceutical purposes are routinely manufactured in a size range from 0.5 to 1.5 mm. The pellet culture provides a similar environment to the natural cartilage (dense cells in avascular hydrogel) and prevents chondrocytes from dedifferentiation.⁵² In addition, this technique is utilized for chondrogenic differentiation of stem cells where a number of cells (2 to 4×10^5) are centrifuged and cultured in a specified culture medium that predominantly contains transforming growth factor β (TGF- β). After 14 days, the cells form a brown nodule-like structure that expresses hyaline cartilage-specific markers such as collagen type II.⁵³

Spheroids are developed from a simple cluster of cells such as embryonic bodies or tumors by 3D culture methods.⁵⁴ Scaffold-free 3D cell spheroids can be obtained from suspension by the floating procedure, suspending drop method or agitation-based methods. Spheroids regenerate the physiological properties of tissues and tumors regarding cell-cell attachment and allow for natural cell-matrix interactions. Such structures can be produced in large numbers and be included in high-throughput screening methodologies.^{55,56} Spheroids can be developed to a size where oxygen and nutrient gradients are similar to the natural tissue.⁵⁷ Spheroids with a radius rate of 200 μm or greater consist of an active layer of proliferating cells on the exterior and quiescent cells on the interior due to nutrient and oxygen carrier restrictions. Remarkably, larger spheroids include necrotic cells at the center which are similar to some solid tumors *in vivo*. Spheroids have been used to assess drug sensitivity and resistance and they are usually more durable to chemo- and radiotherapies in contrast to cells cultured as 2D monolayers.⁵⁸ However, challenges exist when using spheroid models for drug delivery investigations including their formation and holding on identical size, developing spheroids from small number of cells, manufacturing tissue-like spheroids with numerous cell types, evaluating tissue-like spheroids and producing them as suitable models in drug delivery and efficacy testing.⁵⁹ Furthermore, a dissemination gradient along with an enhanced spheroid size and absence of nutrients in the core of the spheroid has been observed.⁶⁰ Cells in the necrotic core lose their functions due to lack of nutrition and accumulation of toxic wastes.⁶¹ These characteristics of spheroids limit drug diffusion to an extent comparable to organs and tissues *in vivo*.⁶²

The use of 3D porous scaffolds has been suggested as a means of replicating the natural properties of the extracellular matrix (ECM) in tissue engineering.

Balancing the attachment of cells to the scaffold with the degradation rates of the scaffolds is a key focus in enhancing tissue formation.⁶³ Hence, a perfect scaffold should have well-determined and reproducible properties so that cells can penetrate, proliferate and differentiate inside the pores.^{64,65} One drawback of using popular 3D scaffolds (such as PLGA-based scaffolds) is that the hydrophobic nature of these materials prevents nutrient diffusion. On the other hand, the pore structure of hydrophilic scaffolds (collagen-based) can be filled via swelling in the culture medium prior to the cells' penetration. Therefore, encapsulation of cells in hydrogels appears to be reasonable.

Hydrogel-based scaffolds have attracted attention because of their optimal composition and structural similarities to natural ECM and their favorable framework for cellular proliferation and durability.⁶⁶ Hydrogels are 3D systems that consist of hydrophilic polymers crosslinked via covalent bonds or maintained together via physical and intermolecular attractions. Hydrogels can accommodate large contents of water or biological fluids and swell without dissolving. The high hydrophilicity of hydrogels is exclusive because of the presence of hydrophilic moieties such as carboxyl, amide, amino, and hydroxyl groups dispersed along the backbone of polymeric chains. Under swollen conditions, hydrogels are soft and rubbery, which is similar to living tissues.^{66,67} One of the main properties of hydrogel organization is that the biomaterials applied should be capable of self-assembly from a liquid monomeric phase to a solid polymeric mesh network.⁶⁸ A hydrogel creates a humid environment that supports cell migration and attracts several exudates.⁶⁹ Hydrogels suffer from a significant limitation in their mechanical strength, prompting the suggestion of chemical and molecular design approaches to enhance their toughness and strength.⁷⁰

El-Sherbiny et al reported challenges dealing with hydrogels including inappropriate cell penetration and uncontrolled cell seeding due to the lack of spatial and temporal control. Several problems are related to the complexity of natural tissues comprising numerous cell types and unique ECM. In contrast, engineered tissues are composed of a single type of cell whose artificial ECM has lower mechanical properties as well as different physical characteristics at both the macroscopic and microscopic levels. This limits their use to soft and non-load-bearing tissues and the absence of intricate microvasculature in most engineered tissues which significantly diminishes the viability and function of seeded cells because of the lack of nutrient transportation and signaling molecules.⁶⁶ Encapsulation of cells in alginate is widely used in laboratories due to its low cost, transparency, biocompatibility and simplicity of cell encapsulation and hydrogel decomposition. Cells suspended in their medium can be mixed with alginate solutions and encapsulated due to ionic substitutions.⁷¹ For example, a

solution of sodium alginate can be added dropwise to a calcium chloride solution to form capsules. In addition, the calcium ions can be chelated to decompose the capsules and release the cells. The main problem with hydrogels is that these structures are mostly composed of a greater than 95% liquid phase, which means that cells have no place for attachment and proliferation. In addition, sample preparation of cells embedded with hydrogel exhibits more technical challenges. Techniques utilized on tissue samples, including mechanical and/or enzymatic disruption, can take advantage through liberating embedded cells but must be performed with utmost care to preserve the integrity of intracellular components while still maintaining adequate yields. The precise properties of the hydrogel must be considered as well.⁷² Another option is to compartmentalize cells into liquefied semi-permeable capsules, which could allow better self-assembled cellular organization and proliferation.⁷³ In such strategies microparticles or other solid elements can be included inside these compartments as supporting elements for anchorage-dependent cells.^{74,75}

The advancement in three dimensional (3D) bioprinting technology promotes reliability, repeatability and adaptability in the fabrication of tissue-engineered scaffolds. Bio-inks containing printable biomaterials, cells and small molecules (cell-laden biomaterials) are being utilized in bioprinter to form a 3D structure in a layer-by-layer fashion.⁷⁶ In order to enhance the capacity of regeneration, bio-inks with similar characteristics to the natural environment of cells are proposed.⁷⁷

Microfluidic and its application

Microfluidics is a well-accepted physics field that is currently used to expand cell biology devices.⁷⁸ By downsizing microscopic systems and taking advantage of accurate processing, high-power biological experiments can be conducted on chips. Particular properties of dynamic flow at the micrometer-scale, spatial regulation of liquid constitution at subcellular resolution, rapid media and temperature variations, and single-cell management and investigation are the main pros of this method.⁷⁹ The aim is not to create a whole living organ, the aim is not to create a whole living organ, but to at least manufacture operational units that carry tissues and organs fundamental roles. The elementary model is a single, perfused microfluidic chamber which includes one type of cultured cell presenting functions of a distinct tissue type. In more intricate patterns, two or more microchannels are linked by permeable membranes that are coated on opposite sides by various cell types to renovate junctions between several tissues.^{80,81} These systems can involve physical powers such as physiologically related levels of fluid shear stress, cyclic strain and mechanical pressure, and permit analysis of organ-specific reactions involving immune cells' response to drugs, toxins or other environmental disturbances.⁸¹

Initially, the innovation in soft lithography and molding of polymers facilitated the development of inexpensive microfluidic tools including open microfluidic devices,⁸² which have further benefits because of the physical properties of these polymers.⁸³ The most prevalent technology for constructing microfluidic devices for use in cell biology is based on the soft lithography of polydimethylsiloxane (PDMS). PDMS is an elastomer that can be built into microfluidic devices via ordinary molding methods.⁸⁴ Its broad application as a suitable material is because of its flexible feature to integrate fluidic gates which are necessary components for main microfluidic use.⁸⁵ Numerous applications of microfluidics in cell-based devices have been acknowledged. As another possible alternative to traditional cell culture and animal studies, human organs-on-chips or microfluidics could change many areas of basic investigation and drug development.⁸¹ Microfluidic chips regulate many system parameters that are not simply managed in 3D static cultures, and help to investigate a wide range of physiological phenomena. They could be used to explore the molecular processes of organ growth and disease and the interplay of the body with stimuli such as drugs, environmental factors, consumer supplies and medical devices.⁸⁶ Microfluidic devices can be used to examine physiological and pathological mechanisms that take place within a relatively short period (approximately <1 month) and rely on comparative cell locations within organ- or tissue-specific microarchitecture.^{81,87} For instance, many aspects of hepatic physiology can be investigated by the use of liver-on-chip applications and include the generation of liver-specific proteins, polarized cell displacement of lipids and other species, and energy metabolism of various patient populations. Stress reactions that include an acute phase reaction as well as ischemia, partial hypoxia or nutritional exclusion can also be evaluated.⁸⁸ On the other hand, the presence of fluid in the microfluidic system leads to the production of physical or chemical gradients, which have been used for noninvasive investigation of cell migration,⁸⁹ cardiac tissue organization,⁹⁰ nerve axon development⁹¹ and metabolic response,⁹² differentiation⁹³ and neurotoxin reactions,⁹⁴ in addition to the evaluation of subcellular construction⁹⁵ and cell-cell junctional cohesion.⁹⁶ Microfluidic systems recapitulating anatomic sites including kidney, intestine, lung, heart, smooth and striated muscle, fat, bone marrow, cornea, skin, blood vessels, nerves, and blood-brain barrier, fat and bone have been investigated over the past few decades. Many of these systems are not considered to be models of organs since only one cell type was developed in one microchannel. Nevertheless, the results of studies indicate that fluid flow and shear stress alone pose a significant impact on cell shape and function.⁸¹ Microfluidics can be used for the thorough study of absorption, distribution, metabolism, exclusion and toxicity of chemicals in vitro, instead of animal models, which is commonly the case in the

pharmaceutical industry.^{97,98} Over the past decade, many researchers have focused on the use of microfluidics to study drug properties and confirm pharmacokinetic and pharmacodynamics modeling, and evaluate drug potency.⁹⁹ This capability allows researchers to directly monitor cell behavior after drug treatment or investigate the spread of local chemical stimuli in the cell.⁷⁹ For instance, a liver microfluidic system showed the interactions of different processes with drugs including drug bio-activation, drug clearance, drug-drug interactions via induction/inhibition mechanisms, sensitivity to drug-induced liver damage, and generation of reactive metabolites that could cooperate with other organs.⁸¹

Overall, microfluidics research is dealing with specific technical challenges. One disadvantage is that bubbles in microfluidic channels may damage cell membranes and prevent the proper function of chips and it is difficult to entirely remove them.¹⁰⁰ Further problems include achieving robust, stable cell seeding in microfluidic channels, banning microbial pollution, and regulating the cell-cell and cell-ECM interactions, all of which should be resolved in order to establish precise tissue structure-function relationships.⁸¹ A fundamental issue that must be improved is the use of an appropriate material for composing microfluidics. Most microfluidics are fabricated from PDMS because of its simplicity, optical transparency, gas permeability and biocompatibility. However, the absorption of serum proteins, small organic materials such as drugs, and high gas permeability can limit the use of PDMS.^{101,102} Stability is another primary constraint. Different factors must align to attain the optimal role of microfluidics over a month or longer, such as cells and ECM coverings, fluidic regulation, bubble elimination and gradient preservation.¹⁰³ Therefore, given the above constraints and recent successes with the use of microfluidics in imitating specific functions at the organ level, this procedure is still in its infancy. However, microfluidics are not expected to be replaced with animal experimentation in the near future because of tough regulatory requirements and complexities in recreating the roles that organs play in the body which researchers are facing. Though with the increasing number of studies about microfluidics, it is possible to gradually replace an animal-based approach at any time.⁸¹

Replicating cell shapes by cell imprinting

Cell functions are regulated by biochemical and biophysical signals that are specific to different types of tissues. This regulation is necessary because cells are situated within distinct spatial microenvironments.¹⁰⁴ In order to understand the process of tissue reconstruction, it is necessary to consider the impact of dysregulation on tissue malfunction and how drug therapy can restore pathologically damaged tissues. This understanding requires a highly simulated environment that replicates the physicochemical factors found in the cellular

microenvironment, including growth factors, ECM proteins, mechanical landscape, and topological cues.^{65,105,106} Based on these factors, several methods have been expanded that partially control biological interactions between cells and their surroundings.¹⁰⁷ The extensive applications of relocating cellular features and molecular patterns on polymer surfaces have recently gained significant attention in the literatures.^{108,109} The presence of microscale and nanoscale topographies induces changes in the morphology of cells, affecting focal adhesion and cytoskeletal tension. Consequently, these alterations lead to modifications in nuclear shape and ultimately influence cell function.^{110,111} Topographic features can be engraved in microparticles that can be combined with cells that will experience such surface signals in a pseudo-3D space.¹¹²

Researchers have found that in addition to chemical recognition, the assessment of the topography of cell-imprinted substrates is also significant. Although adhesion forces mediated by molecular recognition are undoubtedly stronger, weaker binding forces resulting from topography may also become relevant after prolonged contact. It has been observed that cells show a greater inclination to attach to rough surfaces rather than smooth ones.¹¹³ Also, the specifically induced membrane forms, which are finger-printed based on the mature cell types utilized as templates, could regulate the selective activations of genes of the printed mature cells, followed by auto-activation of specific complex cell signaling and metabolomic pathways. Therefore, it seems that cell membranes, owing to their unique proteins and compounds, may play a role in the topographical pattern of the cell-imprinted substrates.¹¹⁴ Fig. 1 demonstrates a schematic view of the cell imprinting process and alteration in cell shapes after cultivation on the imprinted substrate.

Generally, cells are impacted by their environment characteristics which can be explained in terms of chemistry, topography, elasticity or external stimuli. Different cell functions such as adhesion, migration, proliferation or differentiation are affected by the microenvironment and can undergo changes by processes that range from morphogenesis to cancer progression. For example, the effect of mechanical forces on a cell's fate has been extensively investigated. It has been reported that there is a potential for matrix elasticity¹¹⁵ and topography to control the stem cells' fate.^{116,117} Topographical patterns influence membrane and nucleus mechanics through the rearrangement of the cytoskeleton.¹¹⁸ According to a research, the forces applied to integrins are directly transferred (forwarded) to the nucleus, which leads to its stretching and deformation.¹¹⁹ Actin and other intermediate filaments interfere with the transfer of the force and microtubule stability of the nucleus.¹²⁰ Since the nucleus is the largest and most rigid organelle, the tensegrity (tensional integrity) model indicates that it would be the last point affected by morphological deformation or

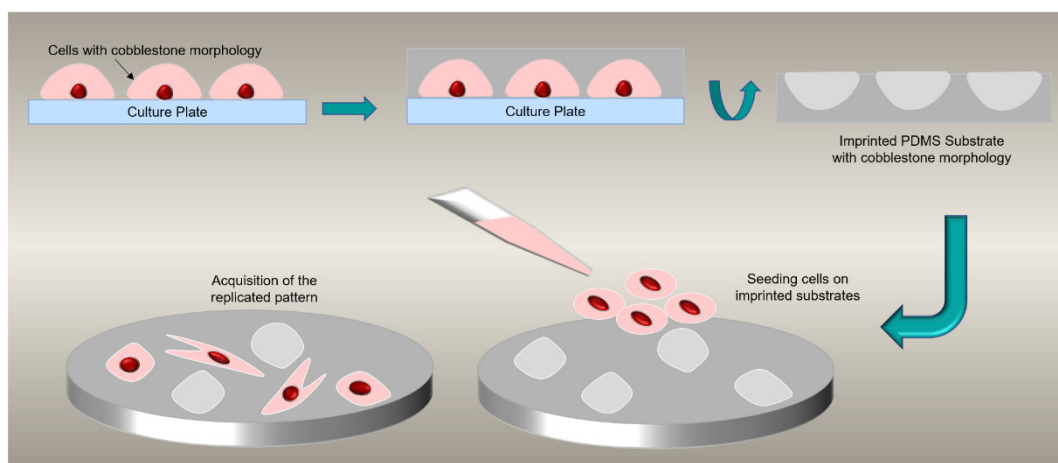


Fig. 1. Schematic view of cell imprinting process. Seeded cells acquire cobblestone morphology on replicated shapes.

mechanical tension to the cell.¹²¹ In addition, it has been confirmed that the nucleus, its nuclear membrane and chromatin are directly connected to the cytoskeleton via the linker of the nucleus-cytoskeleton complex (LINC), the nuclear pore complex and the underlying lamina.¹²² The results of recent studies prove the presence of intrinsic nuclear mechano-transduction pathways, which indicates that the structural formation of the nucleus can sense and react to mechanical tensions. Hence, the nucleus may be a mechano-sensitive structure.¹²³⁻¹²⁵

An important research challenge is the generation of artificial systems which can mimic the detection mechanisms occurring at the molecular level within living systems. The evolution of molecular imprinting provides a valid contribution to this research challenge. With this technology, elective molecular identification sites are presented in a polymer and can therefore mimic the biological properties of the microenvironment. Possible uses of these systems include affinity separations, medical diagnostics, drug delivery and catalysis. Recently, biosensing systems that apply molecular imprinted membranes have become the focus of research in different fields. In these systems, imprinted membranes are being utilized as biomimetic recognition components combined with a transducer element.¹²⁶ According to the “lock and key” process which is similar to natural receptor-ligand interactions as well as easy procurement, molecular imprinting has been extensively used for almost half a century.¹⁰⁹ The first molecular imprinting-based synthetic receptors were introduced by Wulff in 1972 and then this technique became one of the most impressive and adaptable procedures for combining particular molecular recognition sites into a polymeric network to form a receptor-like element. The molecular imprinting mechanism generally includes self-assembly of a template molecule and functional monomers through non-covalent or reversible covalent bonds in order to achieve spatial and matching replication of the form and functionality of the targeted ligand.¹²⁷

Molecular imprinting

Recently, imprinted polymers (MIPs) at molecular level have become a perfect approach to the specific determination of target molecules in complex matrices where other analogous and comparable structural compounds could coexist.¹²⁸ This structure particularly mimics the “lock and key” binding mechanism that occurs in the natural bio-recognition process.^{126,129} In a general sense, MIP as a polymeric system, contains binding cavities in a specific template. Although several fabrication techniques have been suggested over the years, all of them almost follow a similar underlying process which is summarized in Fig. 2. It is demonstrated here that the MIP is acquired by using in situ co-polymerization of functional monomers and cross-linkers around templates (or targets).¹²⁹ During polymerization, the template is integrated into the polymeric matrix and chemical groups of functional monomers are reorganized according to the structure and chemical attributes of the template molecules. The use of the template from the acquired polymeric matrix will form a template complementary recognition site that has high substrate selectivity and specificity.^{126,130} After polymerization, the templates are brought out from the resulting polymeric network (e.g., by cleaning it using a dissolvent), leaving fixed holes of the native template that correspond to its form, size and orientation.¹²⁹ Based on the interactivities between the functional monomer chemical groups and templates, MIPs have been categorized into covalent, non-covalent (e.g., hydrogen bonding, van der Waals or coulomb forces, hydrophobic interactions), or semi-covalent.¹³¹ In covalent MIPs, covalent bonds are organized between the template and functional monomers prior to polymerization; then severed during the template removal phase and reformed during rebinding. Altogether, this process produces cavities that are better defined and more uniformly distributed; however, the needed method is complicated and time-consuming, and because covalent bonds are required, there is a limited selection of monomer–template combinations.¹³² In the

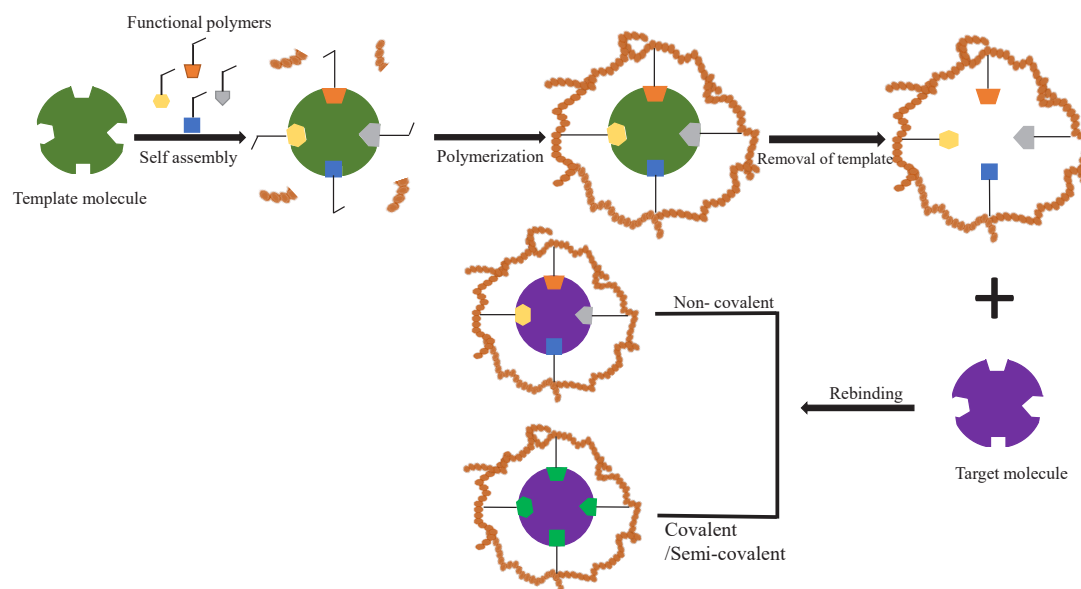


Fig. 2. The schematic view of the MIP procedure.

non-covalent approach, the binding sites are organized through the self-assembly of the monomer and template molecules in the pre-polymerization mixture and are then fixed after polymerization.¹³³ Noncovalent MIPs are relatively simpler to prepare and the templates can be removed easier. However, noncovalent cavities may not be as uniform as covalent cavities. Semi-covalent MIPs combine two previously mentioned techniques, covalent bonds are formed between the template and functional monomer; after polymerization, the template is removed by cleaving these bonds and the analyte non-covalently attaches to its binding sites.^{134,135} (Fig. 2)

Attractive properties of MIPs include mechanical, thermal, and chemical consistency; simplicity of preparation; decreased expense; and receptor-like affinity to targeted molecules.¹²⁷ MIPs are considered to be “artificial receptors” or “plastic antibodies”, which can mimic the biological role of a natural receptor or antibody. Therefore they have the potential to communicate with cells and induce particular cell signaling, intracellular cascades and subsequent modulation of cell actions.

It has been found that one of the most attractive features of natural receptors is their high responsiveness to external stimuli (such as temperature, pH, etc.). Hence, in order to realize an analogy between the synthetic and the natural receptors, stimuli-responsive MIPs (SR-MIPs) have engaged substantial research attention in recent years.¹⁰⁹ The credible and general approach to design stimuli-responsive MIPs is to exploit the molecular imprinting technique in stimuli-responsive materials, which yields the MIPs with the capacity to respond to external stimuli while regulating their affinity for the target molecules and presenting a switchable ability of the binding or releasing activities.¹³⁶ Various studies have concentrated on expanding biological SR-MIPs to imitate

human immune responses such as endogenous stimuli (e.g., reactive oxygen species, variations in physiological pH and temperature, overexpressed protein and enzyme levels) and exogenous stimuli (e.g., temperature, light, changes in the magnetic field, etc.). Also, the recognition ability of SR-MIPs in changes in the environment to simulate dynamic antigen-antibody or bioreceptor-ligand interplay in an organism has been investigated.^{136,137}

However, the primary consideration in MIPs is predominantly focused on its use in adsorption, separation sensing and catalysis of molecules.¹²⁷ In order to identify a cell, MIPs should have a specific binding affinity to special cell membrane molecules (e.g., proteins, lipids and glycans) or the cell membrane assembly.^{138,139} Therefore, approaches for MIPs that mediate cell recognition are commonly placed into two categories¹²⁷: 1) cell membrane-molecular imprinting and 2) whole cell imprinting procedure. Cell membrane-molecular imprinting generally refers to the imprinting of specific parts of cell membrane molecules such as glycan chains and epitopes of membrane proteins.^{140,141} In addition, imprinting a bioactive ligand (e.g., proteins or peptides) that has specificity to cell membrane receptors could allow for indirect cell identification.¹⁴² However, for cell-membrane-molecular imprinting, the selection of suitable cell membrane molecules throughout the imprinting procedure is critical to the ultimate efficiency of the cell's recognition capability.¹²⁷

This procedure is appropriate for imprinting microorganisms like bacteria and viruses as they are comparatively stiff. Cell diagnosis based on the whole cell imprinting scheme relies not only on the cell-like cavities but also on the numerous non-covalent interactions between MIP matrices and cell membranes. Together, these two imprinting strategies have complimentary

benefits that enable excellent feasibility and cooperation in the design of MIPs for selective cell recognition.¹²⁷ The combination of the said two techniques may provide a structure with similar features at both the molecular and cellular scales.

Molecular imprinting

Nanostructured materials in MIPs

With the focus on molecular imprinted technology, MIPs with various forms and scales have been promoted so far.¹⁴³ In recent years, great advances have been achieved in the production of functional nanomaterials and researchers have assigned themselves to investigate MIP nanomaterials (diameters less than 200 nm) with the same particle size and controllable morphology. Compared to conventional MIPs, MIP nanoparticles (MIP-NPs) exhibit a great dispersibility, high surface-to-volume ratio and easy rinse of template molecules, also having a high binding capacity, high selectivity, high affinity and excellent water compatibility.¹⁴⁴ Hence, MIP-NPs applications are currently being expanded, certainly for biomedical separation and detection. This includes using them for bioimaging,^{145,146} diagnosing cancer,¹⁴⁷ delivering drugs to specific targets¹⁴⁸ and releasing drugs in a controlled way.¹⁴⁹ MIP-NPs are anticipated to become a precious research tool for many different kinds of biomedical studies. Molecularly imprinted nanogels (MIP-NGs) are also a promising innovative material in the biomedical field. MIP-NGs as swollen hydrogel nanoparticle networks formed from either hydrophilic or amphiphilic polymer chains. They have a few key characteristics such as being stable composition, size change ability and low toxicity, stable in serum and capable of effectively encapsulating drugs. Because of these qualities, they are known as advanced systems for drug delivery. So, MIP-NGs can be very useful in disease diagnosis, delivering drugs to treat cancer and capturing images of inner parts of the body.¹⁵⁰ Currently, MIP-NMs' applications are extended in biomedicine since they can be selective and effective same as natural receptors. In the past few years, the molecules used to make nanomaterials are not exclusively comprised of small molecules, but also biological macromolecules (like proteins), cells different microorganisms, viruses, etc.¹³⁶ For example, Zeng et al fabricated MIP-NPs with an affinity to the hydrophilic peptide GFP-9 by inverse microemulsion polymerization. The surface has a spot where peptides can stick easily and can strongly attach to the template molecule GFP-9. For imprinting short peptides, the key point is to use it as an epitope to achieve the respective macromolecular recognition.¹⁵¹ In another study, Teixeira et al discovered that growth factor- β 3 (TGF- β 3) MIP-NPs produced through epitope-imprinting reverse microemulsion polymerization, can identify and selectively attach to TGF- β 3 even in complex human fluids (platelet lysate). Furthermore, adipose-derived stem cells were cultivated with MIP-NPs and incubated with platelet

lysate resulting in an increased collagen II-rich matrix. This offers a cost-efficient, reliable and easily expandable substitute for non-living growth factor molecules to direct cell fate. In addition, it was confirmed that MIP-NPs can serve as GF chelating ligands to control cell activity and the operative surface of MIP-NPs can selectively identify and seize TGF- β 3 and 3D cell cultures using MIP-NPs, which can enhance the accumulation of chondro-related matrix components.¹⁵²

There are numerous techniques for preparing MIP-NMs for small biological molecules that are imprinted like in-site polymerization, surface imprinting, precipitation polymerization, suspension polymerization and so on. Each approach possesses distinct benefits and drawbacks like the straightforward preparation and appropriate execution of precipitation polymerization. Furthermore, the resulting MIPs exhibit uniformity in their structure and are effortlessly adjustable in terms of their size. However, the template molecules enclosed within them are not readily extractable. Furthermore, if the desired compound is specifically identified in the mixture, the remaining template molecule in the MIPs could detach, leading to potential inaccuracies in the experiment. Hence, while getting ready MIP-NMs, the particular employed approach relies on the actual circumstances.^{136,153}

Cell imprinting

Cell imprinting is a technique that replicates cell shapes using cells as a mold to produce substrates with cavities analogous to the original cellular morphology. Different types of hydrophobic or hydrophilic materials are suggested for this process. Hydrophobic materials such as PDMS are stable at room temperature and can physically replicate microscale and nanoscale features due to the molecular structure of its backbone (e.g., Si=O). It is proposed that in hydrophilic materials such as polyacrylamide or methacrylated gelatin (GelMa), the molecular interactions between functional groups (carboxyl and amine) of materials and cells play a role in the imprinting process, which might provide an increased affinity. However, the possible evaporation/absorption of water or molecules may damage its features and interfere with the outcome. Another essential criterion for material selection is elasticity. The effects of substrate stiffness on cell expansion have been extensively evaluated.¹⁵⁴ PDMS is well-known for its tunable stiffness and surface properties, ability to mold, optical clearness, gas permeability and lack of toxicity.¹⁵⁵

Nevertheless, the hydrophobic surface of PDMS prevents impressive cell adhesion to the uncoated PDMS surface. The results of studies have shown that special receptors on cell membranes can sense alterations in ECM hardness. The physical characteristics of the ECM are recognized by integrins that connect ECM to the actin cytoskeleton inside the cells and transfer it to the nucleus through the LINC complex to induce nucleus rotation and reorientation. The signals are translated by changes

in nucleus conformations and respond by gene or protein expressions.¹⁵⁶⁻¹⁵⁸

In a general cell imprinting procedure, cells are freshly isolated from tissues, cultured on polystyrene dishes, and fixed at the highest confluency that does not sacrifice the cell's original phenotype. PDMS substrates are subsequently prepared by mixing a silicon base elastomer and curing agent. The fixed cells are used as a mold to make a replicate with prepared PDMS. The solid form of PDMS is separated and utilized as a new substrate for further analysis.^{159,160}

This is a simple, safe and user-friendly process and the key element of this protocol is the preparation of cell-based molds that have the highest confluency and lowest changes in the original phenotype. This imprinted substrate could be used as a substitute for conventional tissue culture polystyrene-based dishes.¹⁶¹

Cells cultured on imprinted substrates

The cell membrane has intricate chemical and topographical features that are frequently identified as flexible and textured patterns. Nevertheless, the smooth and stiff transparent polystyrene (PS) tissue culture plates extensively applied for in vitro cell cultures show different physical properties as compared to ECM.¹⁶² Despite numerous endeavors to improve the connection of cells that are attached to PS plates, these substrates are orders of magnitude stiffer than soft tissues.¹¹⁵ The results of previous studies indicate that PDMS, at a 1:10 ratio of curing agent, induces more osteogenicity in stem cells than polystyrene plates.^{163,164} In some studies, engineering approaches such as lithography have been used to generate substrates with 3D features that mimic physiological shapes and patterns similar to native tissues. For example, aligned microgrooves are fabricated by photolithography to resemble the physiological dimensions of the ECM structure in tendons, muscles, nervous and cartilage tissues.¹⁶⁵ Engineered topographical substrates which imitated natural topographies have been used to regenerate whole tissues in vitro, such as corneal substitutes¹⁶⁶ and vascular graft.¹⁶⁷ However, the mechanism underlying cells' response to topography is not yet fully clear at the cellular level. Recently, there have been remarkable studies showing that topographical factors including size, shape and geometric features can have important effects on many cell behaviors such as adhesion, migration, alignment and differentiation.¹⁶⁸⁻¹⁷⁰ Among these factors, topography size seems to play an important role in modulating cell behavior. Although the cell reaction to topography is different and depends on the cell type, the effect of topographical size (such as width, distance and depth of surfaces) exists for all cell types. Whereas microtopography (such as size greater than 10 μm , which is the length scale of a mammalian cell) mostly influences entire cell morphology, nano topography is included especially with subcellular detecting

instruments. The relation between the size of topography and cellular sensing organelles has been shown in various investigations. Most importantly, topographic features that have an optimal size as the most potent inducer of cellular response, have been mostly present in both the length scales. For instance, a basic surface roughness value of 1.1nm was recognized to influence the rate of proliferation for murine osteoblasts.^{171,172} In conditions of roughness higher than this value, the rate of cell proliferation decreased. A threshold distance of 73 nm between the nanoislands attached to the adhesive ligand was also observed to regulate the integrin clustering process and then monitor the adhesion of MC3T3 osteoblasts on the nanotopographic surface.¹⁷³ At the micro length scale, human corneal epithelial cells are able to change their alignment on grooved substrate when the pitch is larger than a threshold range of 0.8–1.6 μm .¹⁷⁴ In this manner, such data concerning the importance of topographical size would be supportive in planning for appropriate materials and platforms in tissue engineering and regenerative medicine usages.¹⁷¹ In Table 1, cell imprinting is compared with other cell culture methods.

In addition, it has been shown that surface topography features can influence cellular drug uptake. As previously mentioned, biological science research and drug evaluation are mainly based on 2D cell culture techniques, which are unable to provide a reliable relevant physiological environment.^{185,186} According to studies, binding to the integrin-targeted cell-adhesive peptide RGD (Arg-Gly-Asp) segments plays a role in cell interaction with the bio-imprinted substrate of cells in vitro. It has also been discovered that cancer cells changed their $\beta 1$ integrin expression, cell morphology, the organization of their cytoskeleton and proliferation when cultured on cell imprinted substrate surfaces in comparison with a 2d surface.^{142, 187} Therefore, a suitable substrate can have significant effects on drug susceptibility, gene expression and protein synthesis by having the chemical composition, topographical and mechanical properties of the substrates.¹¹ In a study conducted by Shahriyari et al the role of cell imprinting on viability and drug susceptibility of breast cancer cells to doxorubicin (DOX) was investigated. According to their study, the response of MCF7 cells to DOX was monitored for 24 hours. Although the biocompatibility was increased in the imprinted substrates, the cultured cells were more sensitive to the drug as compared to the plain substrates. MCF7 cells on imprinted PDMS and GelMA substrates demonstrated respectively 37% and 50% higher cell death as compared to the corresponding plain PDMS and GelMA. In addition, the cells on the imprinted hydrogel showed about 70% more drug sensitivity than the cells cultured on the imprinted PDMS substrates. Therefore, they concluded that multiscale cell membrane topography can mediate intracellular signaling and drug uptake.¹¹ Also in another study Domura et al.

Table 1. Advantages and disadvantages of different cell culture approaches

Cell culture method	Advantages	Disadvantages
Imprinting on a chip	Monolayer/all cells are accessible Safe in signaling Wide range of applications (tissue engineering or cancer research, routine cell cultures) Capable of producing realistic in vitro models More reliable/predictable/repeatable A valuable tool for personalized medicine Induction of both physical and chemical cues Improved cell-to-cell interactions compared with other 2D cultures ¹⁷⁵	Need specific facilities to produce the chips Lack of standardized platforms ^{175, 176}
Polystyrene plates	Monolayer/all cells are accessible Simple/priceless Popular/standardized ¹⁷⁷	Totally irrelevant to the native tissues Induction of wrong signals to cultures Cells lose their phenotype ¹⁷⁸
Alginate hydrogels	3D hydrogel Simple/priceless Popular/standardized ¹⁷⁹	Chemically irrelevant to the native tissues Low cell attachment and proliferation due to the presence of a substantial amount of water ¹⁸⁰
Traditional imprinting	Monolayer/all cells are accessible Simple/priceless Safe in signaling ¹⁸¹	Low efficiency/unpredictable/unrepeatable Lack of standardized platforms ¹⁸¹
Aggregate/ pellets/ organoids/ spheroids	3D culture More accurate representation of in vivo cell-to-cell and cell-to-ECM signaling ¹⁸²	Difficult in cell expansion Difficult in oxygenation -Transiently resemble cell organization and interactions Difficult to maintain long-term cultures ^{182, 183}
Scaffolds/ 3D bio-printing	High reproducibility Co-culture ability Chemical-physical gradients ¹⁸⁴	Lack of vasculature Challenges with cells/materials Difficult for cell analysis ¹⁸⁴

stated that the morphological spreading factors (nucleus/cytoplasm area ratio) caused by the natural substrates are related closely with the cellular proliferation and the IC50 drugs of two different types of breast cancer cells (MDA-MB-231 and MCF-7).¹⁸⁸ Tan et al. also investigated the role of paclitaxel and doxorubicin on the expression of caspase 3, the expression of proliferating nuclear antigen (PCNA), the number of cells and the secretion of vascular endothelial growth factor (VEGF) on endometrial cancer cells cultured in PDMS substrate. Based on their study, a culture substrate with cell-imprinted topography, which has nano- and micro-resolution, is able to regulate the response of endometrial cancer cells to chemotherapy drugs. Their results revealed that the topography affected the cell reactions in a drug-dependent manner. So the topography-related susceptibility of paclitaxel and doxorubicin effects varied. A culture substrate with cell-imprinted topography, which has nano- and micro-resolution, is able to regulate the response of endometrial cancer cells to chemotherapy drugs. Hence, it seems that the physical architecture of the cancer cell and the components of the nano- and micro-environment may be an appropriate prospective target to increase the clinical activity of traditional drugs. Furthermore, studies' results imply that the cells discriminate between the various cell-like topographies (positive and negative bioimprints), suggesting that a practical topography is most desirable as

a growth platforms in experiment design.¹⁸⁹

Maintenance or restoration of the original phenotype (redifferentiation of dedifferentiated chondrocytes)

Regulation of cell function with a reliable and low-cost method is still regarded as a significant challenge. In fact, the fabrication of a substrate with simple features (grooves, ridges or pits) and basic geometries may not provide enough signal to mimic the complex structure of natural ECM. In addition, the characteristic features and design should be optimized for each cell type.¹⁹⁰ In a similar manner, a substrate that can recreate the natural niche of the target cells can initiate particular signals driving a phenotypic change.¹⁹¹ For example, Mahmoudi et al. utilized cell-imprinted substrates to induce chondrogenesis, proposing that stem cells react to the design of local chondrocytes imprinted on their culture substrate.¹¹⁴

Although autologous chondrocyte implantation (ACI) is an approved procedure used to repair cartilage lesions, this method has several limitations such as the patient's age (<45 years) and size of the lesion (<6 mm). In addition, ACI cannot be used for patients with osteoarthritis. During the past 20 years, numerous research endeavors were made to improve ACI procedures; however, there was less progress reported for the cellular part. Chondrocytes lose their original phenotype once isolated

from the body and cultured *in vitro*.¹⁹² The spherical morphology of the natural chondrocytes changes to a spindle-like morphology and there are changes in their specific marker expressions, mostly in collagen II to collagen I. In order to prevent dedifferentiation, a special culture medium supplemented with TGF- β and 3D cultures such as alginate encapsulation that can maintain the chondrocyte phenotype *in vitro* has been proposed. TGF- β , like most growth factors, has no or limited approval for clinical applications due to the risk of side effects.¹⁹³ For example, palifermin is a keratinocyte growth factor (KGF) prescribed for severe oral mucositis patients after chemotherapy. Chondrocyte imprinted substrates can maintain the original chondrocyte phenotype or restore the phenotype in dedifferentiated chondrocytes. Hence, dedifferentiated chondrocytes that have fibroblast-like morphology and collagen type I expression could re-differentiate to a spherical morphology and collagen type II expression after cultivation on a chondrocyte imprinted substrate.¹⁹⁴

Imprinting offers several advantages over growth factors in terms of safety, stability, and reproducibility of outcomes. While growth factors are highly effective molecules, they can potentially expire or lose their functionality. In contrast, imprinting has the ability to physically generate signals that regulate cell function. However, no existing literature compares the effectiveness of growth factors to imprinted substrates. Additionally, it is important to consider the potential undesirable signals that may result from the combined use of growth factors and imprinting. Therefore, it is crucial to optimize the amount of growth factor when incorporating physical signals.¹⁹⁵

One main problem in autologous cell transplantations is the shortage of accessible cells. Expansion of normal cells *in vitro* changes their original phenotype because of alterations in their environment.¹⁹⁶ In terms of phenotype, the dedifferentiation of chondrocytes is characterized by a decrease in the expression of specific markers, including collagen type II, aggrecan, and transcription factor SOX9, and an increase in the expression of fibroblastic markers such as collagen type I and versican.¹⁹⁷⁻¹⁹⁹ The significance of this is crucial in the field of cartilage tissue engineering as dedifferentiated chondrocytes tend to transform into fibroblasts rather than producing hyaline cartilage, which is necessary for the functioning of articulating joints.¹⁹⁹ Therefore, the loss of a chondrocyte phenotype prior to use in experimentation or implantation is of utmost importance for tissue engineering.

Induction of differentiation in stem cells

Regardless of the extensive attempts to accurately control stem cell fates with engineered patterned substrates, a trustworthy and inexpensive method that regulates stem cell behavior outside the body remains to be developed. The ECM has a complex regular structure and the

construction of simple geometries that contain grooves, ridges, dots or pits cannot adequately recapitulate the ECM architecture. Designing an optimal topography for all cell types requires an extensive effort.¹⁹⁰

It is apparent that changes in the shape of a nucleus will change the cell's fate.^{158,200} The nucleus is linked to the cytoskeleton and it is presumed that alterations in nuclear topography at the nanoscale level are induced by the cell's shape. In a recent study by Kamgouyan et al, an analysis using atomic force microscopy was performed on imprinted substrates derived from five different types of cells: osteoblasts, tenocytes, stem cells, fibroblast-like cells, and C28 cell lines. The findings of the study revealed that there were unique nanopatterns observed in the roughness parameters including amplitude, spatial, and hybrid for each individual cell type.²⁰¹ Therefore, the effect of the geometry of the nucleus on contacts between chromatin fibers and their adjustments should be investigated.^{122,202}

Table 2 enlists published studies that pertain to cell imprinting. Researches have shown that cell imprinted substrates can induce chondrogenic,^{114,194} keratinogenic,¹⁵⁸ osteogenic,²⁰⁰ tenogenic,²⁰³ neurogenic,²⁰⁴ myogenic²⁰⁵ and Schwann cell²⁰⁶ differentiation in stem cells. In addition, Abadi et al reported improvements in cardiomyocyte differentiation when induced pluripotent stem cells (iPSCs) were cultured on cardiomyocyte imprinted substrates.²⁰⁷

Bonakdar et al assessed the differentiation, redifferentiation, and transdifferentiation of chondrocyte imprinted substrates. They used the cell imprinted approach to probe stem cell differentiation and sought to determine the commitment of stem cell differentiation to the desired phenotypes. In order to display the potential of cell imprinting to recapitulate the ECM architecture, researchers investigated chondrogenic and tenogenic differentiation and teno-chondro transdifferentiation on a PDMS template of corresponding cells. The results showed that with this simple imprinting procedure, both the nanoscale and microscale aspects of the cell topography and their main phenotypes were transferred to the substrate in a way that the spindle morphologies of semi-fibroblasts and adipose-derived stem cells (ADSCs) were changed into a spherical morphology when cultured on chondrocyte imprinted substrates. The spindle morphology of tenocytes transformed into a spherical morphology when the tenocytes were cultured on chondrocyte imprinted substrates. Based on the results of gene expression analysis, particular genes can be up-regulated when cells are cultured on imprinted substrates. The significant increase in collagen type II expression (as a specific marker of chondrogenic differentiation) in both ADSCs and semi-fibroblast cells indicated the success of the imprinting approach. Similar results were obtained from tenogenic differentiation of a tenocyte imprinted substrate in another study and specific genes for tenocytes such as tenascin, decorin and tenomodulin were adjusted

Table 2. Summary of studies on cell imprinted substrates.

Year	Author	Description of the study	Ref.
2012	Jeon and Kim	Bone mineralization of osteosarcoma (MG63) on MG63 imprinted patterns	208
2013	Mahmoudi et al	Chondrogenic differentiation of rabbit adipose-derived stem cells (ADSCs), changes in cell morphology	114
2014	Mashinchian et al	Keratinogenic differentiation of human ADSCs, modeling the nucleus	158
2014	Lee et al	Myogenic differentiation of human mesenchymal stem cells (MSCs) using UV curable poly(urethane acrylate)	205
2016	Bonakdar et al	Tenogenic differentiation of stem cells, redifferentiation, and trans-differentiation of tenocytes	194
2017	Wang et al	Replication of cancer cells for cell recognition	209
2018	Kamgouyan et al	Osteogenic differentiation of human ADSCs, coating of hydroxyapatite on imprinted surface	200
2018	Farvadi et al	Nanoparticle uptake on fibroblast imprinted substrate	210
2019	Kavand et al	Conductive cell imprinted substrate based on polydimethylsiloxane (PDMS) and carbon nanotube	211
2019	Kavand et al	Using chondrocyte as molding template for direct cell photolithography	165
2019	Moosazadeh et al	Schwann cell differentiation of rat ADSCs	206
2020	Gholami et al	Osteogenic differentiation of MSCs on osteoblast imprinted substrate and regeneration of calvarial bone in rats using collagen-based scaffold	212
2020	Haramshahi et al	Tenogenic differentiation induction of the tenocyte imprinted substrate was greater than the tissue replica	213
2020	Ghazali et al	Neuronal differentiation of a human stem cell line using ReNcell [®]	204
2021	Kashani et al	Chondrogenic differentiation on a chip and regeneration of cartilage in a rabbit model of hyaline cartilage	214
2021	Kamgouyan et al	Determination of nanoscale fingerprints of imprinted substrates	201
2021	Kashani et al	Computational fluid dynamics (CFD) simulations on HUVEC, L929 and chondrocyte-imprinted-based integrated microfluidic device	215
2021	Dadashkhan et al	Induction of Schwann cell differentiation in human MSCs cultured on imprinted substrates and exposed to β -carotene	216
2022	Nazbar et al	Improving stemness in ADSCs cultured on imprinted substrates	217
2022	Babaei et al.	Improving cell adhesion, proliferation and osteogenic differentiation in imprinted substrates coated with bone-extracted proteins	164

for ADSCs cultured on a tenocyte imprinted substrate.²⁰³ In another research, stem cell imprinted substrates were utilized in the long-term expansion of ADSCs to maintain stemness. In addition, downregulation in the expression of specific markers in osteogenic and adipogenic differentiation was observed for stem cells seeded on imprinted substrates that were exposed to the differentiation chemical signals.²¹⁷

The cell imprinted method provides a monolayer structure that enables cells to develop while maintaining their primary phenotype (Fig. 3). Furthermore, the utilization of growth factors such as TGF- β , FGF, BMP, and IGF, which are commonly suggested for chondrogenesis, is reduced by employing this approach.²¹⁸ On the other hand, it has been shown that high doses of chemical stimulants may have an inhibitory instead of stimulatory effect. For instance, synovial fibrosis, osteophyte formation, cartilage degeneration and bone remodeling have been reported for altered signaling in TGF- β content.²¹⁹ Growth factors can assist in expanding proliferation and postponing chondrocyte dedifferentiation. However, their effective concentration in cultures is consistently altered, that means they do not completely prevent phenotypic changes.²²⁰

Haramshahi et al. reported that tenogenic differentiation potency (scleraxis and tenomodulin expressions) of

tenocyte imprinted substrates was higher than the imprinted tendon tissue and lower than BMP12 treated groups.²¹³ Therefore, it is valuable to know the synergistic effect of growth factors (or chemical substances) and cell-imprinted substrates on cell/stem cell functions. It can be predicted that cell imprinted substrates may result in the reduction in the effective concentration of growth factors or decrease the exposure times.

Babaei et al, showed improvement in cell adhesion, proliferation and osteogenic differentiation by coating the osteoblast imprinted substrates with bone extracted proteins.¹⁶⁴ In another recently published report, it was found that small molecules such as β -carotene can enhance the differentiation capacity of cell imprinting.²¹⁶

Overall, the mechanisms of stem cell differentiation induced by physical stimuli and the maintenance of cell phenotype are not realized, though a number of key mechanotransduction components including focal adhesions, cytoskeletal contractility, Rho GTPase signaling and nuclear regulation have been demonstrated to be involved in the force-mediated differentiation.^{221,222} The cytoskeleton of cells is a dynamic structure, meaning that cells can actively respond to physical signals by adjusting their mechanical properties through the remodeling of cytoskeletal components. This dynamic response of the

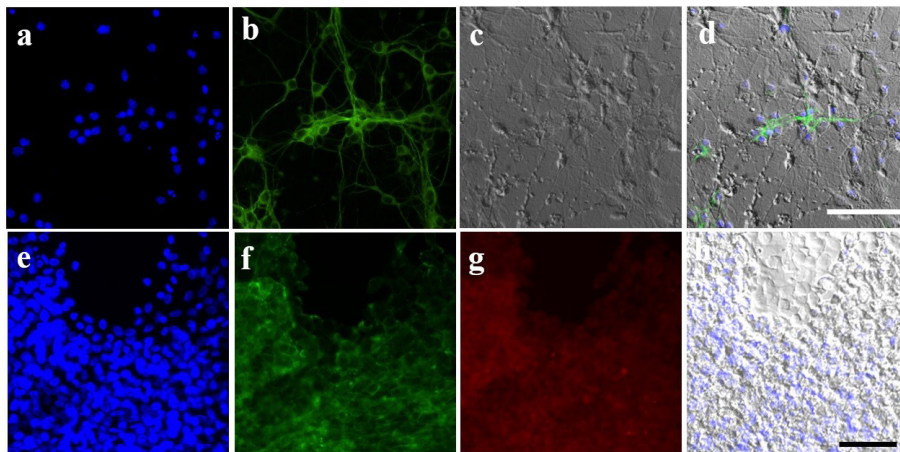


Fig. 3. Stem cell differentiation on imprinted substrates. a-d) Mouse adipose-derived stem cells (ADSCs) cultured on neuroblast imprinted substrates. a) Nucleus stained with Hoechst, b) actin stained with phalloidin, c) optical image of stem cells and imprinted shapes, and d) merged images. e-h) Human ADSCs cultured on chondrocyte imprinted substrates. e) Nucleus stained with Hoechst, f) actin stained with phalloidin, g) immunostaining of collagen type II, and h) optical image of imprinted shapes.

cytoskeleton not only affects cellular contractility but also triggers molecular signaling pathways, specifically the activation of RhoGTPases. Consequently, these pathways regulate intracellular processes and control gene expression.²²³

Cell modeling of diseases in vitro

Various models, both continuum and discrete, is granted to depict the aggregate cell behaviors in connection with challenges relating to disease modeling. Normally, discrete cell modeling employs quasi-infinite or boundary less 2D lattices to model collective cell behaviors in Petri dish-like environments. Modeling the entire behavior of cells within in vitro culture environments is a complex subject that is generally carried out by separate cell models, typically cellular automata. Recently, in a particular area of modeling cell behavior, cellular automata have been used for modeling cell adhesion and proliferation.²²⁴

An ideal model should imitate all-natural cues in in-vitro condition, including autocrine, paracrine and endocrines as well as physical internal or external signals (e.g., forces, patterns, or electromagnetic waves), which seems impossible to simultaneously consider thousands of factors. In addition to these complexities, genetic diversity, age or nutrition should be added.²²⁵ Therefore, simplifying models alongside eliminating factors to the utmost possible extent is rational. Because it has been proven that cell imprinted substrates could mimic the natural topography of cells in vitro, these substrates can be appropriate choices. For example, during the disease progress, both the cell phenotype and expression undergo changes. These cells can be isolated and cultured in vitro to impart imprinted substrates that have exact patterns. Subsequently, adult or embryonic stem cells can be cultured on these imprinted substrates to determine the changes in protein and gene expressions when the cells are exposed to varying concentrations of different drugs and different time points (which is difficult and unethical

in animal models). The most important advantages of cell imprinted substrates in disease modeling include the stability of the model and accessibility to the development of the disease during a short period.^{114,226,227}

Various models have been developed to assess diseases in vitro. The appearance of lab- and organ-on-a-chip tools confirms that the information obtained from 2D cell cultures, in Petri dishes, varies significantly from the outcomes obtained from more biomimetic microfluidic environments that were created from interconnected chambers and channels. Therefore, organs-on-chips have a tremendous potential to analyze basic processes of organ physiology and disease. They are well-adapted to the investigation of biological events that rely on tissue microarchitecture and perfusion that include comparatively acute cases of less than one month pathophysiological processes.⁸¹ For instance, dynamic alterations of oxygen tension have been applied in organs-on-chips to mimic disease states, including heart ischemia²²⁸ or vaso-occlusion in sickle-cell disease as a result of polymerization of hemoglobin S in deoxygenated erythrocytes.²²⁹ Based on similar studies, the use of low levels of fluid shear stress similar to those within the collecting ducts and proximal tubules of the living kidney caused elevated differentiation (e.g., epithelial cell polarization, formation of primary cilia), developed molecular and drug delivery roles, and created more in vivo-like toxicity reactions when primary rat,²³⁰ dog²³¹ and human²³² cells obtained from these tissues were cultured in chips.

Human diseases can easily be investigated in a widely governable environment by using in vitro disease models after the production of characterized human embryonic stem cell lines. The proposing of human-induced PSCs (iPSCs) a decade ago, paved the way for in vitro research on different diseases.²³³ The full pluripotency of iPSCs has been explained by various researches via the most accurate experiment of pluripotency (i.e. tetraploid

complementation), and revealed the feasibility of obtaining pluripotent iPSCs from somatic cells.²³⁴ Due to these properties, iPSCs have multiple biomedical benefits to fundamental studies, drug screening, toxicological studies, disease modeling and cell therapy.²³⁵ There has been tremendous progress in disease modeling with the increase of iPSC-derived organoids.²³⁶ The soluble and biophysical signs utilized to instruct organoid differentiation from iPSCs have been increasingly purified to produce complex 'tissues in a dish'.²³⁷ Besides, researchers have been able to produce specific tissues from organs such as the brain and gastrointestinal tract.^{238,239}

In addition, microvascular network remodeling includes angiogenesis, determined as the germinating of new capillaries and network modeling related to the formation and linkage of available vessels. For instance, blocking remodeling would be useful in numerous pathologies, including cancer, proliferative retinopathies and rheumatoid arthritis. In other diseases such as myocardial infarction, stroke and high blood pressure, strengthening a novel procedure is quite desirable. Improvement of such therapies converges a further comprehension of each sub-process association with microvascular remodeling besides our understanding of how each sub-process is coordinated across a network.²⁴⁰

Alternative methods provide an alternative to conventional strategies and animal models, and they could solve many issues of basic studies and drug development. Since cell imprinted substrates can imitate the natural cell phenotype, researchers can observe different cell viability/toxicity when exposed to substances and cultured on their respective imprints as compared to polystyrene plates.²¹⁰ The applications of the cell imprinting method are shown schematically in Fig. 4.

Cell therapy

Cell therapy is an approach where cellular components are injected or transplanted into a patient. Throughout the world, many products are presently under extensive analysis and their market is anticipated to grow quickly in the near future.²⁴¹ Some of the drugs are still present in the form of organic or protein substances to relieve symptoms, not for definitive remedy. However, cell transplantation procedures can be considered to be the frontiers of new technology for fighting against diseases. Cell therapy products utilize cells as a drug. The cells are often obtained from a patient's own tissues in order to obtain individual attributes by manipulation and culture, and are subsequently re-injected into the patient. Hence, cell therapy products are served in personalized medicine as compared to drugs that can be administered to everyone.²⁴² Cell therapy approaches can be closely linked to gene therapy, cancer vaccines, drug delivery, tissue engineering and regenerative medicine.²⁴³ The very basis of these therapies is bone marrow transplantation in cancer treatment.²⁴⁴ They can potentially be used

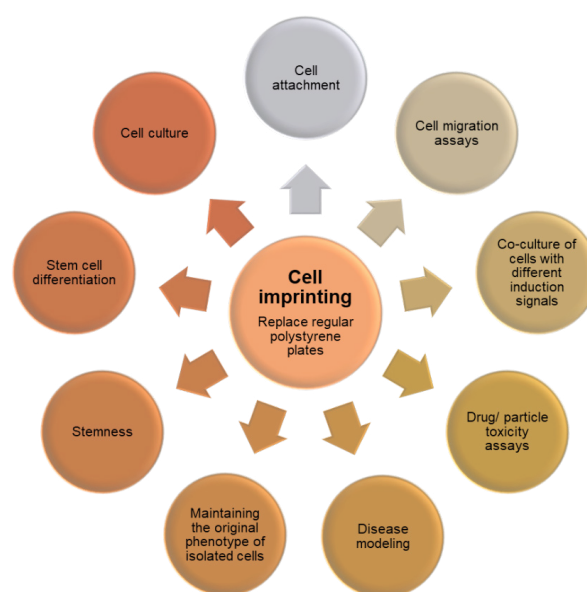


Fig. 4. The different applications of cell imprinting approach

for cardiac diseases (myocardial infarction and heart failure),²⁴⁵ diabetes mellitus,²⁴⁶ bone and joint disorders,²⁴⁷ genetic disorders²⁴⁸ and skin wounds.²⁴⁹ Although each disease has intricate specific features, the basic principles of cell therapy in these cases are almost the same.²⁵⁰

Cell therapy is aimed to repair malfunctions rather than to generate a new tissue, which could bring along duplicity and unfavorable consequences. Different types of cells can be applied to heal the defective tissue such as resident stem cells, multipotent adult progenitor cells or embryonic stem cells. There are various procedures that include the use of both autologous (from the same individual) and allogeneic (from another individual of the same species) progenitors of adult or embryonic stem cells and different trophic factors such as VEGF, TGF, EPO or HGF, which aim to stimulate proliferation and differentiation of resident stem cells.²⁵⁰

Despite its vast benefits, problems exist with cell therapy that should be solved. The most critical limitations of stem cell therapy are safety issues, including tumorigenicity and immunogenicity. Although the risks are considerably lower in cell (non-stem cell) therapy, the improvement in the quality of a patient's life after cell transplantation should be guaranteed.²⁵¹

Cell imprinted substrates can induce physical signals for the redifferentiation of phenotypically altered cells in vitro. Considering the increased safety of the induction by physical cues rather than chemical cues, cell imprinting can be used for many kinds of cell therapeutic applications in which the source of normal juvenile cells is not available in an older patient. Because the potency of normal cells is reduced in an older person, it might be desirable to isolate cells from an older patient and culture them on imprinted substrates derived from young brain-dead donors or fetal-derived cells. In addition, for therapeutic procedures such

Review Highlights

What is the current knowledge?

✓ For cell-based experiments involved in research or clinics, an artificial ECM is required to support cell function, which can be considered as a substrate.

✓ To better mimic the natural environment in vitro, factors such as forced polarity, flattened cell shape, mechanical/biochemical signals, and subsequent cell-to-cell communication must be regulated.

What is new here?

✓ The various 3D cell culture techniques such as organoids, pellets, spheroids, 3D scaffolds and hydrogels can be used as simulate in vivo conditions for cell growth.

✓ The cells show a greater inclination to attach to rough surfaces rather than smooth ones.

✓ It is apparent that changes in the shape of a nucleus will change the cell's fate.

✓ Cell imprinted substrates can induce physical signals for the redifferentiation of phenotypically altered cells in vitro.

✓ Imprinted polymers are considered to be artificial receptors or plastic antibodies, which can mimic the biological role of a natural receptor or antibody.

as autologous chondrocyte transplantation that require millions of cells through expansions, cell-imprinted substrates could be a desirable substitute for conventional tissue culture polystyrene plates. However, the proposed concepts should be researched in the future. Recently, Kashani et al fabricated a chip with cell-imprinted topography at the bottom to culture stem cells under a dynamic culture for 14 days. The differentiated stem cells by the chondrocyte imprinted substrate could regenerate hyaline cartilage in a rabbit model when compared to undifferentiated stem cells.²¹⁴ This finding was important since it was previously reported that undifferentiated stem cells could not regenerate meniscus tissue after transplantation.²⁵² In other words, in order to regenerate tissues, pre-stimulation of stem cells is required prior to transplantation.

Conclusion and future perspectives

Cell imprinted substrates could be used to investigate the molecular processes of organ growth and diseases by novel methods in vitro apart from the interplay of the body with drugs, environmental agents, consumer products and medical tools. The idea of modeling disease by cell imprinting should be analyzed and compared with studies on animal models in the future. In addition, it is suggested that both physical and chemical signals should be combined for better simulation of the natural environment. Surface coating of the cell imprinted substrate with ECM molecules such as collagen or the addition of small molecules to the medium used to culture the cells could be considered as a subject of future research.

As illustrated in the current study, microfluidic chips with cell-imprinted patterns can be fabricated and utilized for dynamic cultures. Improvements in cell imprinting techniques can pave the way for pre-differentiation of stem cells towards the desired phenotype through a safe, efficient, repeatable and reliable approach.

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Competing Interests

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