Current Trends and Challenges in Biointerfaces Science and Engineering

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Abstract

The cellular microenvironment is extremely complex, and a plethora of materials and methods have been employed to mimic its properties in vitro. In particular, scientists and engineers have taken an interdisciplinary approach in their creation of synthetic biointerfaces that replicate chemical and physical aspects of the cellular microenvironment. Here the focus is on the use of synthetic materials or a combination of synthetic and biological ligands to recapitulate the defined surface chemistries, microstructure, and function of the cellular microenvironment for a myriad of biomedical applications. Specifically, strategies for altering the surface of these environments using self-assembled monolayers, polymer coatings, and their combination with patterned biological ligands are explored. Furthermore, methods for augmenting an important physical property of the cellular microenvironment, topography, are highlighted, and the advantages and disadvantages of these approaches are discussed. Finally, the progress of materials for prolonged stem cell culture, a key component in the translation of stem cell therapeutics for clinical use, is featured.

INTRODUCTION

The phenotypic and epigenetic characters of biological cells are defined by their native environment and respond to external factors in a myriad of different ways. Most prominently, changes in the microenvironment can lead to adaptations of cytoskeletal organization and cellular gene expression. More macroscopically, changes in the microenvironment of a biological cell can lead to cell adhesion, proliferation, or apoptosis. Critical cues defining the cellular microenvironment can be categorized as biological, chemical, and physical (1). Although substrate-induced cell changes almost always involve specific interactions with the extracellular matrix (ECM), or at least ECM components, other important cues exist. Known physical cues include elasticity, the local micro- and nanostructure, and gradient distributions, as well as gross topological features (2). With an emerging understanding of the biological processes that define the cellular microenvironment in vivo, there is a growing opportunity for fundamental research into the mechanisms and processes involved in cellular sensing and signal processing. Similarly, there are ample opportunities for synthetic surfaces and materials with fully defined chemical and physical properties to play major roles in developing novel clinical concepts, such as regenerative medicine (3, 4). In fact, the availability of defined model surfaces and materials is often considered a prerequisite for multiparametric studies needed for delineating between the plethora of complex biological processes occurring in parallel. This review focuses on important physical and chemical aspects of the cellular microenvironment and the transdisciplinary opportunities that are arising because of the integral convergence of biology, chemistry, and materials science.

CHEMICALLY DEFINED SUBSTRATES

Most cells must solidly adhere to a substrate to engage in their typical biological interactions and to exhibit their typical phenotypes. However, cell adhesion not only is dependent on the interplay of different cell surface receptors but further requires appropriate spatial and temporal control of the ligands (5). Many of the apparent differences between in vitro and in vivo cultures can be linked to qualitative and quantitative discrepancies in the interfacial presentation of cell-binding ligands. As a part of a tissue, biological cells undergo a three-dimensional organization that allows them to receive and respond to directional cues. However, most in vitro models are two-dimensional and lack this three-dimensional cell organization.

This rather simplified approach is in part technologically motivated: Although a range of different technologies exist for flat surfaces to provide precise levels of control, microstructuring in three dimensions is substantially more challenging. Flat surfaces can be modified using a myriad of techniques, including photolithography (6), electron beam lithography (EBL) (7), or soft lithography (8). Importantly, these techniques can resolve biological, physical, and chemical features with micrometer- and nanoscale precision. For example, a property as simple as roughness can define the adhesion and alignment of endothelial cells (9).

Most of the micropatterning methods exploit spatially controlled chemical reactions of previously generated functional groups or, alternatively, activate the local chemistry of a surface in spatially controlled ways to selectively immobilize bioactive ligands. An example for the first approach is the vapor-based coating of a substrate with a reactive polymer film followed by the spatially controlled immobilization of a biotin ligand. EBL of a polystyrene substrate followed by spatially selective physisorption of fibronectin may serve as an example for the second approach. In the latter case, EBL is used to render specific areas of the surface hydrophilic, which then alters the physisorption of proteins, such as fibronectin. Although this approach is experimentally

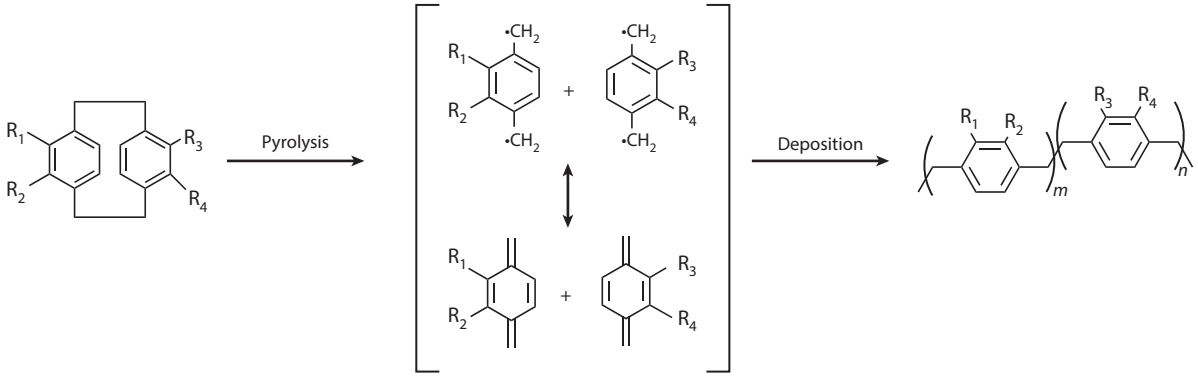
simple, it is rather unspecific in that it does not allow for discrimination between different types of ligands or proteins. If more specific immobilization of one or multiple ligands is required, covalent immobilization can provide a powerful alternative. One or multiple types of chemical groups are introduced onto a surface so that they bind selectively to their target ligand. Ideally, there is no cross-reactivity with any other ligands that may be present in the solution. If these criteria are fulfilled, the chemical groups are often referred to as chemically orthogonal. Substrates that provide multiple chemical anchor groups with orthogonal reactivity are of particular interest.

Self-assembled monolayers (SAMs) of alkanethiolates are versatile substrates for surface modification. SAMs form spontaneously on coinage metals, such as gold, and can be prepared with a wide range of different chemical end groups. In addition, SAMs are compatible with several different micro- and nanopatterning methods. However, SAMs are intrinsically hampered by their limited stability in physiological environments. In addition, they are restricted to gold and other noble metals as substrates. SAMs of alkanethiolates on gold have been used to attach biomolecules and cells for the study of cell/biomolecule material interactions (10, 10a). In a particularly instructive example, Koepsel and coworkers (11) used microfluidic microstructuring techniques to micropattern SAM-based stem cell culture substrates. In this study, the substrates varied with respect to ligand identity and density. First, hydroxyl-terminated oligo(ethylene glycol) alkanethiolate was coated onto a gold substrate. Next, a poly(dimethylsiloxane) (PDMS) device with several microchannels was brought in contact with the SAM-modified gold surface. Taking advantage of the limited stability of SAMs, a solution containing $NaBH_4$ was allowed to flow over certain areas of the gold surface to remove the alkanethiolate monolayer. Finally, the free surface regions were coated with a second alkanethiolate that provided chemical anchor groups for the immobilization of peptide ligands, such as Arg-Gly-Asp-Ser-Pro (RGDSP). Human mesenchymal stem cells (hMSCs) were cultured on these substrates and preferentially attached to areas patterned with the RGDSP peptide.

Fundamentally, the separation of surface and bulk properties can greatly contribute to the diversification of the immobilization chemistries, while ensuring that a proven immobilization method can be applied in the same way to a diverse set of substrate materials. This will eliminate the tedious reconfiguration of the experimental procedures when switching between different substrate materials. In the case of SAMs, such an approach involves the deposition of a thin gold coating on the substrate of choice. Among the polymer coating techniques, vapor-based processes combine several features uniquely suited for biointerfaces. Vapor-based coatings do not require solvents or plasticizers and therefore often have good biocompatibility. In addition, vapor-based coatings typically maintain micrometer- or nanometer-sized topologies present in the bulk structure (12). Still, only a limited range of chemical vapor deposition (CVD)-based polymerization processes are known (12–16). For example, Tenhaeff & Gleason (12) have used plasma polymerization to prepare ultrathin polymer coatings. The same group has also pioneered a very promising technology known as initiated CVD (iCVD). In the case of iCVD, microstructured substrates can be obtained through prepatterning of the substrate with an initiator (17, 18).

Although an increasing number of vapor-based polymerization processes exists to date, CVD polymerization of [2.2]paracyclophanes using the Gorham process has been the most frequently used approach for vapor-based coatings. The resulting polymers are poly-*p*-xylylenes (19), and the unsubstituted and chloro-substituted derivatives are known as parylene N, C, and D. In particular, these technically proven polymers have found broad utility for packaging of semiconductors and biomedical devices alike (20–22).

Although the parylene coatings are well-suited as inert packaging or to alter barrier properties, they fail to provide chemical anchor groups needed for the design of the cellular microenvironment



Scheme 1

Chemical vapor deposition (CVD) polymerization of substituted [2.2]paracyclophanes to prepare functionalized poly(*p*-xylylenes).

or the immobilization of biological ligands (23, 24). More recent work is thus focused on the synthesis of reactive CVD coatings, i.e., poly(*p*-xylylenes), with one or multiple types of chemical anchor groups appropriate for subsequent polymerization (**Scheme 1**).

The actual CVD process is an adaptation of the Gorham process that is well-established for conventional parylene coatings (19): A functionalized [2.2]paracyclophane is sublimated under vacuum (0.2–0.3 Torr) in a first heating zone and then transferred into a second heating zone, which is maintained at a constant temperature between 600°C and 800°C. Under these conditions, hemolytic cleavage of the paracyclophane bridges results in reactive monomers, while preserving the chemical anchor groups. The monomeric units are quinodimethanes, which spontaneously polymerize onto a substrate of choice, as long as the substrate is cooled between −40°C and 40°C. The synthesis of [2.2]paracyclophanes with a wide range of functional groups (25–29) can result in chemically distinct polymer coatings with different anchor sites (30). An example of a CVD system is shown in **Figure 1**.

A library of chemically reactive CVD coatings has been prepared by the modified Gorham route from substituted [2.2]paracyclophanes (**Scheme 2**). For biomedical applications, CVD-based reactive polymers feature a range of beneficial properties (**Table 1**). During the Gorham process, predominantly linear polymers are formed. If two or more paracyclophanes are copolymerized using the CVD technology (31, 32), multifunctional films with orthogonal reactivity for further immobilization of multiple distinct ligands are obtained. Furthermore, CVD polymer films are solvent resistant and show excellent adhesiveness to a wide range of different substrates, such as gold, glass, or the elastomer PDMS.

With respect to biomedical surface modification, the introduction of highly efficient chemical reactions has been a hot spot of recent research activities (33). In particular, click reactions are widely used for their fast reaction kinetics, high selectivity, specificity, and near-quantitative yields (34). Several reviews have been published describing the opportunities of click reactions for materials science and biotechnology (33, 35–37). In many cases, the limiting step is the introduction of adequate functional groups to facilitate the immobilization of ligands via click chemistry. In this respect, CVD coatings have a particular appeal. The utility of CVD coatings for click chemistry has been highlighted by alkyne-functionalized CVD coatings that were functionalized with azide-modified ligands (28). The CVD process facilitated Huisgen-type click reactions on substantially different substrates, such as glass, plastics, stainless steel, paper, and silicon. Identical results were

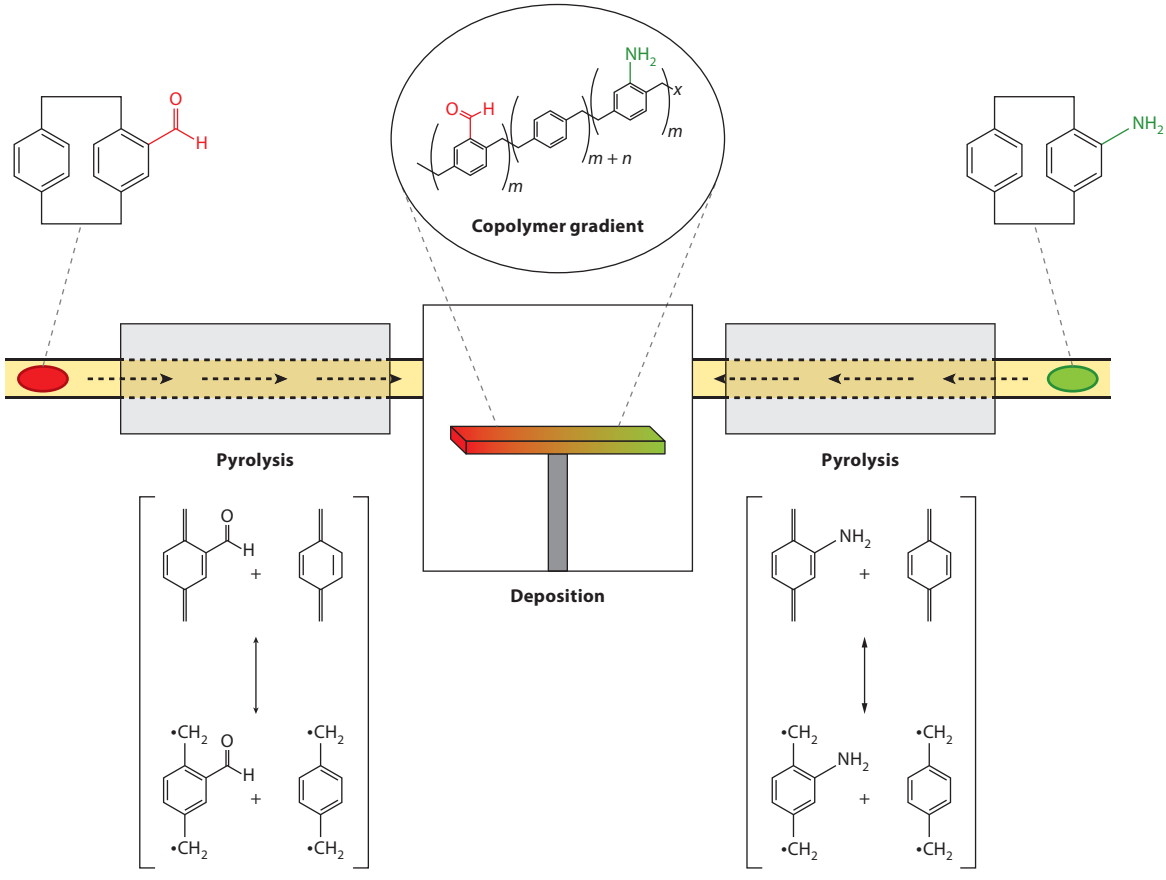


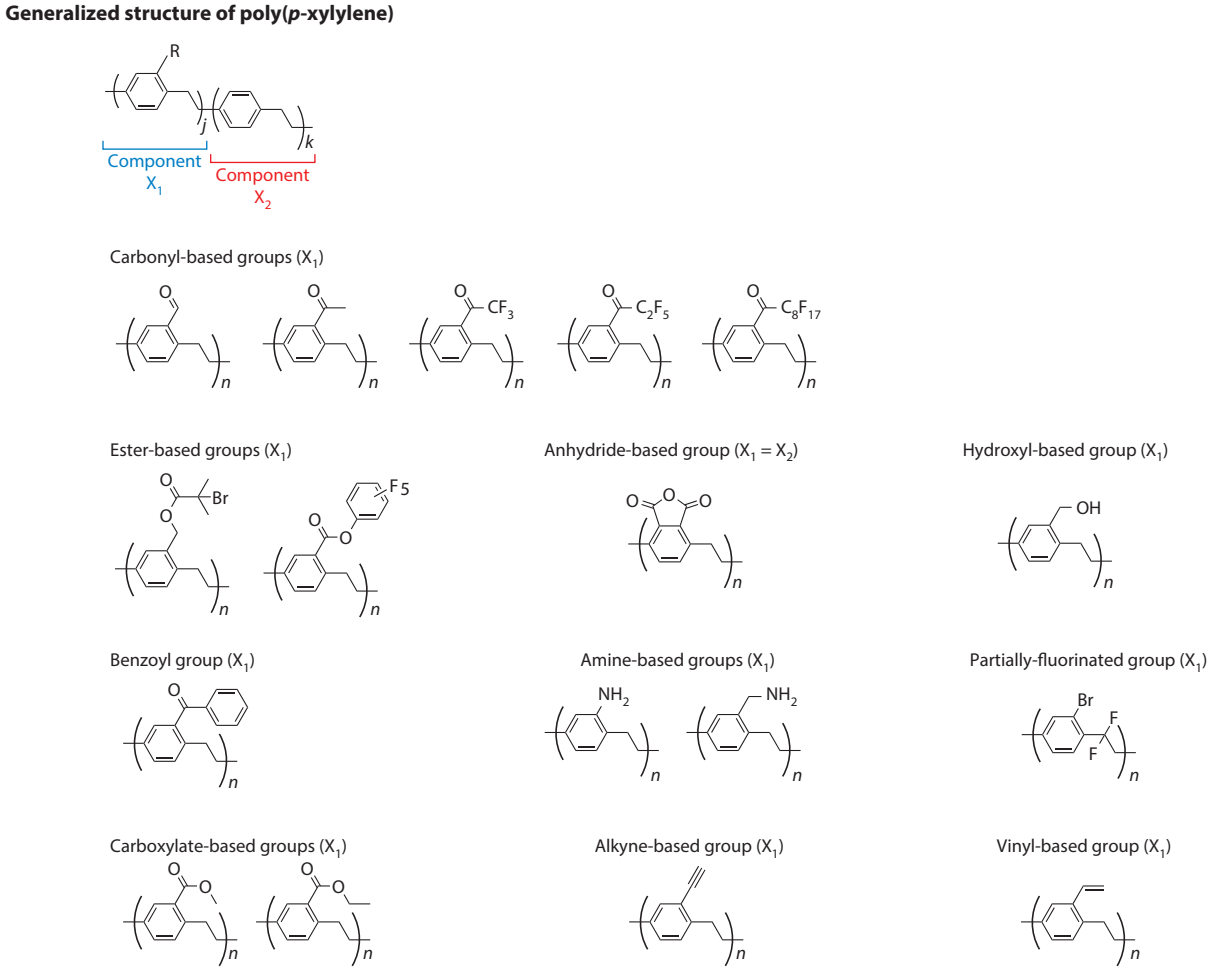
Figure 1

Schematic illustration of the system installation used for chemical vapor deposition (CVD) (co)polymerization to prepare reactive coatings as well as polymer gradients. Adapted with permission from Reference 30.

achieved without the need to adjust the modification protocols when switching from one substrate to the other.

Similarly, hydrazones can be formed via reaction of aldehydes with a hydrazine or hydrazide derivative. Similar to the Huisgen-type click reactions, this immobilization reaction proceeds rapidly at room temperature and with high specificity. Using CVD polymerization, Nandivada et al. (29) prepared poly(4-formyl-*p*-xylylene-*cop*-xylylene) films and modified them with bioactive ligands (**Figure 2**). First, the starting material 4-formyl[2.2]paracyclophane was homolytically cleaved and deposited to yield a nanometer-thick film of poly[(4-formyl-*p*-xylylene)-*co*(*p*-xylylene)]. Then microcontact printing (μCP) enabled the spatio-selective immobilization of adipic acid dihydrazide prior to incubation with the bioactive ligands. Distinct patterns were readily apparent after incubation, confirming selective adsorption of the bioactive moieties. As in many other examples of the CVD polymerization of substituted [2.2]paracyclophanes, the functional groups were preserved, and no side reactions were detected.

In a further extension of this work, the reactive coating poly(4-trifluoroacetyl-*p*-xylylene-*cop*-xylylene) was deposited in PDMS microchannels (38). The chemical reactivity of the



Scheme 2

Examples of vapor-based reactive coatings based on functionalized poly(*p*-xylylenes).

trifluoroacetyl groups toward hydrazide-modified ligands was excellent and yielded homogeneous binding throughout the entire microchannel.

In addition to the above-mentioned approaches, biomolecules are often immobilized via native amino groups. To pursue immobilization using primary amino groups, reactive CVD coatings were developed that had either anhydride or pentafluorophenol ester groups as anchor sites (39). **Figure 3** shows an immobilization scheme that uses pentafluorophenol ester films (26). The synthesis of the functionalized [2.2]paracyclophane is rather straightforward and uses the commercially available [2.2]paracyclophane as a starting point (39). In contrast, [2.2]paracyclophane 4,5,12,13-tetracarboxylic dianhydride is best synthesized by acid-catalyzed condensation of 4,5,12,13-tetrakis(methyloxy carbonyl) [2.2]paracyclophane. The latter molecule can be prepared by Diels-Alder reaction of acetylenedicarboxylic acid methyl ester with hexatetraene in good yields (40).

Table 1 Comparison of chemical vapor disposition (CVD) polymer coating with other techniques

	System	Stability	Uniformity	Thickness control	Chemistry control	Substrate dependency	Process time	Cost	Scalability
CVD polymer coating	Vapor	Good	Good	Good	Good	Low	Medium	Medium-high	Medium
Dip coating	Solution	Good	Medium-poor	Medium	Medium	Medium-low	Fast	Low	Good
Spray coating	Solution	Good	Medium	Medium	Medium	Medium-low	Fast	Low	Good
Plasma polymerization	Vapor	Good	Good	Good	Poor	Low	Medium	Medium-high	Medium
Langmuir-Blodgett film	Solution	Poor	Medium-good	Good	Good	High	Slow	Medium	Low
Self-assembled monolayer	Solution	Poor	Good	Good	Good	High	Slow	High	Low

SURFACE MICROSTRUCTURING

In many applications, spatially controlled surface modification is desirable. Already, patterned substrates have found diverse applications in biotechnology, more specifically, in biosensing, tissue engineering, and drug discovery (41). Depending on the microstructuring technique, functional feature sizes can range from nanometers to millimeters (23, 42). In principle, surface patterns can be obtained by masking the substrate during deposition of the polymer, as can be implemented for CVD techniques. Alternatively, homogeneously coated substrates can be micropatterned by spatially controlled immobilization. Because of the short contact times and the needed compatibility with highly sensitive proteins and peptides, the reactions should proceed with high reactivity and under physiological conditions. Versatility with respect to different patterning techniques is another important factor. Click reactions are ideally suited for these applications. **Table 1** summarizes different surface microstructuring techniques that are suitable for biomaterials.

ELECTRON BEAM LITHOGRAPHY

EBL is an ultrahigh-resolution patterning technique that was originally developed for the semiconductor industry. EBL is a top-down fabrication technique for direct writing of features ranging from 10 nm to 100 nm. In recent years, EBL has been successfully adapted by biology for micropatterning of biomolecules and cells (50). EBL requires high vacuum and uses expensive equipment, such as a focused electron beam (51). Compared with other lithographic methods, the undisputed benefit of extremely small feature sizes and precision is undisputed, but it can be offset by the high fabrication cost and expensive equipment. In addition, EBL, being a direct writing method, is relatively slow compared with other patterning methods. Nevertheless, EBL has found impressive applications in studies related to adhesion (7), differentiation (52), and the controlled growth and organization (53) of a range of different cells. For example, a biodegradable polyester, polycaprolactone, was modified via EBL with a disordered nanopit pattern. This particular microstructure was found to influence the differentiation of osteoblast progenitor cells

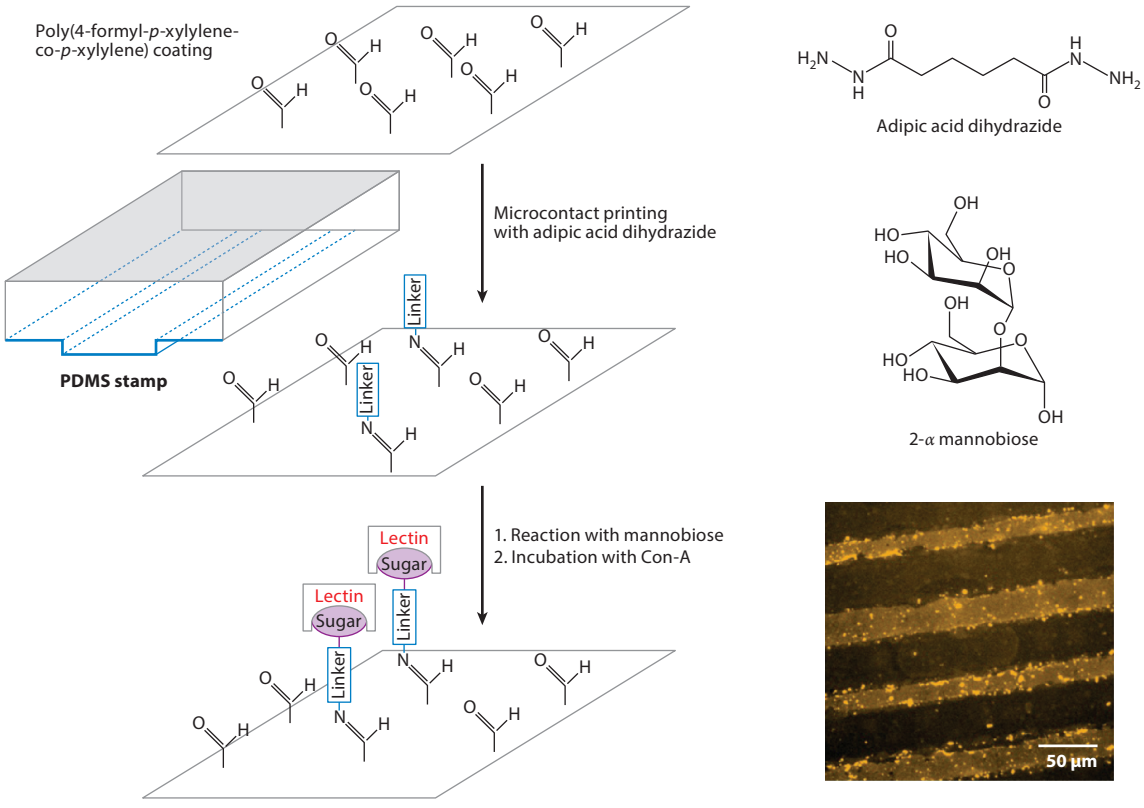


Figure 2

Immobilization based on [(4-formyl-*p*-xylylene)-*co*(*p*-xylylene)] coating and the corresponding fluorescence micrograph after protein immobilization. Adapted with permission from Reference 29. Abbreviation: PDMS, poly(dimethylsiloxane).

(52). Similarly, EBL can be used to modulate the interactions and networks of neural cells (53). Polymer hydrogels, such as poly(amidoamine), can be micropatterned via EBL to guide protein adsorption. When neuronal cells were cultured on these microstructured substrates, spatially controlled cell adhesion and subsequent neurite outgrowth into the interconnects resulted in the formation of an intercellular neural network (**Figure 4**). The use of EBL for microstructuring of polymer hydrogels can facilitate the creation of complex patterns for single-cell analysis, which are critical for understanding important physiological and pharmacological effects.

PHOTOLITHOGRAPHY

Photolithography is another interface microstructuring method that initially came from the semiconductor industry (23). Because of the lower operational cost associated with photolithography, it is currently one of the mainstream techniques for the creation of protein and cell patterns (54–56). As for EBL, patterns are generated when features from a mask, the master, are transferred to a substrate of choice by exposure to UV light. To date, most of the photomasks are composed of optically transparent polymers that are economically prepared using computer-aided design software. Photolithographic microstructuring can be used to create features between one and hundreds of micrometers over large surface areas (57). Depending on the feature sizes,

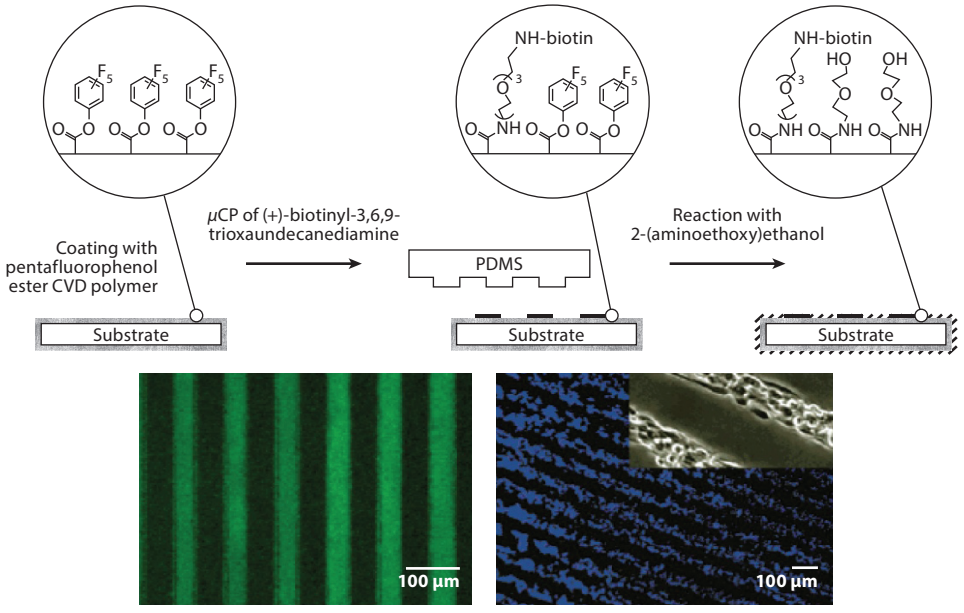


Figure 3

Immobilization based on pentafluorophenol ester containing chemical vapor deposition (CVD) polymers. A poly(dimethylsiloxane) (PDMS) stamp was used for μCP to generate patterned ligand onto polymer, and the fluorescence micrograph shows self-assembly of fluorescein-conjugated streptavidin (*left*). Fluorescence micrograph of bovine aortic endothelial cells and antibody-modified polymer surface (*right*). Adapted with permission from Reference 26.

photolithographic processing still requires clean rooms. Another potential disadvantage is that photolithography is less suited for processing of substrates that already contain biological ligands. More recently, alternate photolithographic methods have emerged that obviate the need for clean room facilities, such as microscope projection photolithography (MPPL) (58).

Photolithographic microstructuring has been demonstrated for a plurality of substrates ranging from metals (59) and glass (60) to polymers (61) and hydrogels (62). For example, the low-cost

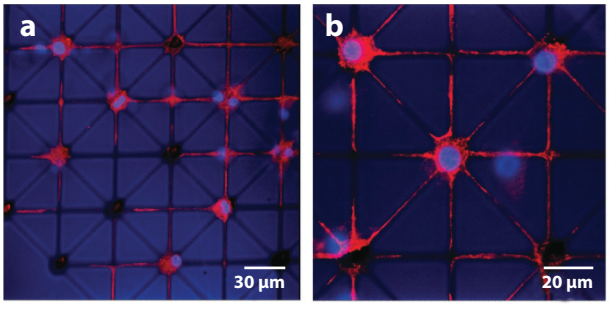


Figure 4

Confocal microscopy of neurites grown on electron beam lithography patterned microwell networks (10-μm diameter) connected by microchannels (1-μm width). Cells were treated with NGF (for 48 h) and immunostained with DAPI (cell nuclei, *blue*), FITC antivinculin antibody (focal contacts, *red*), and TRITC phalloidin (actin filaments, *red*). (*a*) Cell growth and interconnection between neurites (*b*), single cell in one microwell and extension of neurites along microchannels. Adapted with permission from Reference 53.

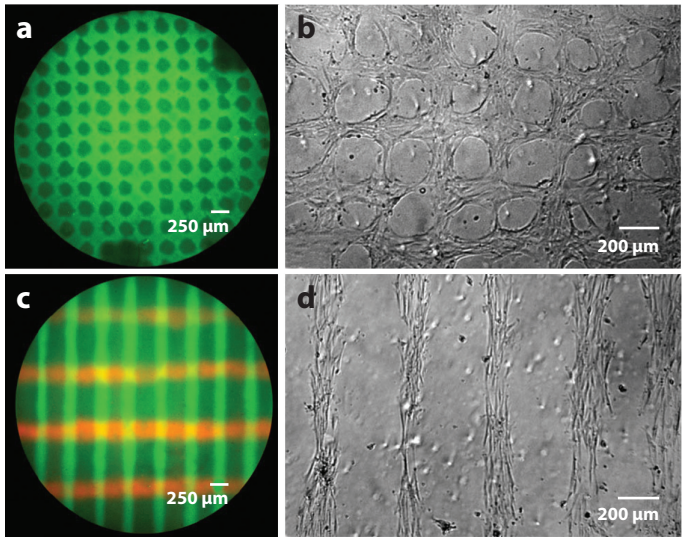


Figure 5

Fluorescence of poly(ethylene glycol)–diacrylate (PEGDA) hydrogels patterned with (*a*) ACRL-PEG-RGDS peptide (*green*). (*c*) ACRL-PEG-REDV peptide (*red*) and ACRL-PEG-RGDS peptide (*green*). (*b*,*d*) Phase contrast of human dermal fibroblasts (HDFs) attached to the surface of the ACRL–PEG–RGDS patterned hydrogels. Note that in panel *d*, HDFs have bound to RGDS patterned regions but not to REDV patterned regions, as expected. Adapted with permission from Reference 62.

patterning of poly(ethylene glycol) (PEG)-diacrylate (PEGDA) hydrogels can be achieved without the use of a clean room. In addition, the lower resolution can tolerate less-expensive masks, which in many cases can be created using standard ink-jet printers (62). This low-cost approach resulted in the modulation of peptide immobilization and the adhesion behavior of human dermal fibroblasts (HDFs) as a function of UV exposure time. Specifically, the unspecific adhesion peptide RGDS and a second, endothelial cell–specific adhesion peptide (REDV) were immobilized on the PEGDA hydrogel. The photoimmobilization scheme took advantage of a monoacryloyl-PEG-peptide derivative that was applied to the hydrogel surface along with the photoinitiator. Subsequent UV exposure through a transparency resulted in spatio-selective conjugation of the peptide derivative. The interfacial peptide concentration was found to be a linear function of the UV exposure time. Interestingly, HDF adhesion occurred only on the RGDS patterned regions but not on the REDV areas, confirming the biological activity of the immobilized peptides (**Figure 5**).

Photolithography is, however, not limited to soft matter per se. For example, micropatterns of titanium, aluminum, vanadium, and niobium were prepared via photolithographic microstructuring to explore protein binding and adhesion of human bone cells (63). In a first step, a silicon wafer was coated with a base layer of one of the metal oxides. Next, a positive photoresist was applied and developed through exposure to a mercury lamp. Subsequent removal of the undeveloped photoresist and the deposition of a second metal oxide layer resulted in a patterned substrate. This process yielded various features, including stripes and dots with an average width of 50 µm, 100 µm, and 150 µm (63). The affinity of osteoblasts to the various metal oxides was then assessed, and it was found that, irrespective of the material combination, aluminum was the least biologically favored substrate for osteoblast adhesion.

Using infrastructure that is already routinely found in a life sciences laboratory, a particular version of photolithography, MPPL, exploits the fluorescent light from a microscope for

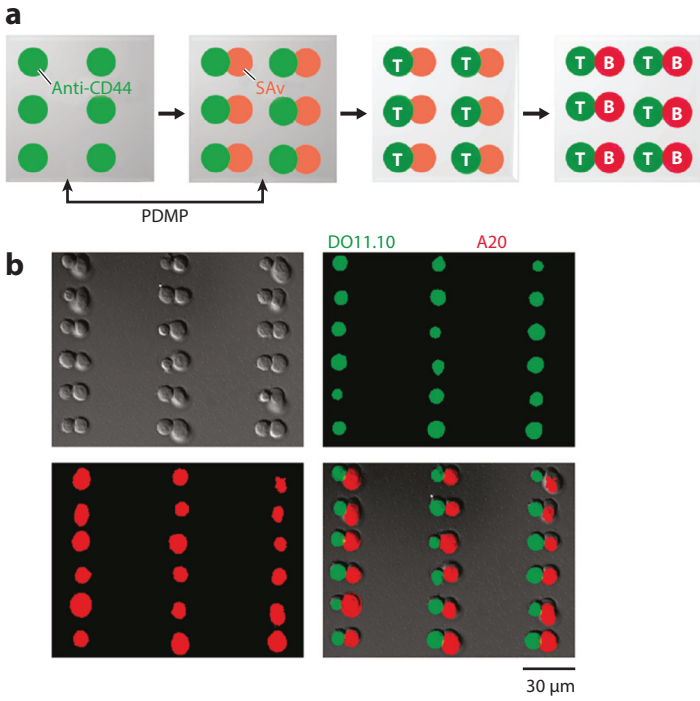


Figure 6

Cell array generated via photolithography. (*a*) Schematic of T/B cell 2D array fabrication. (*b*) Representative images of T/B array. DIC (*top left*), green fluorescence (*top right*), red fluorescence (*bottom left*), and DIC/green/red overlay (*bottom right*). Adapted with permission from Reference 65. Abbreviation: PDMP, poly(2,2-dimethoxy nitrobenzyl methacrylate-r-methyl methacrylate-r-poly(ethylene glycol) methacrylate).

photopatterning. By using MPPL, multiple proteins and cells can be microstructured in one projection step (64). This study takes advantage of a custom-synthesized biophotoresist, poly[2,2-dimethoxy nitrobenzyl methacrylate-r-methyl methacrylate-r-poly(ethylene glycol) methacrylate] (PDMP), which degrades upon exposure to UV light under physiological conditions. Using PDMP, researchers prepared protein arrays: A base substrate was homogeneously coated with a biotin layer. Next, the PDMP was deposited on top of the biotinylated surface and patterned via MMPL. The PDMP was locally removed, and the biotin was exposed for binding the protein streptavidin (65). This process was repeated twice more to ultimately create an array of three proteins. In a further extension, the multi-protein arrays were used to coculture two types of immune cells. DO11.10 $CD4^+$ T cell blasts were patterned by using a biotinylated antibody followed by the immobilization of biotinylated A20 lymphoma cells (**Figure 6**). These and similar cell arrays are of particular interest for studies of immunological synapses between T cells and antigen-presenting cells.

SOFT LITHOGRAPHY

Soft lithographic patterning refers to a series of methods for printing and molding with elastomeric polymers, which is experimentally simple (8). Typically, an elastomeric polymer stamp is casted from a replica mold (66). The efficient transfer of information from the master to the elastomeric

stamp requires a master with sufficient rigidity to allow for separation of master and mold. The masters can be microstructured to take on a wide range of geometries and patterns. The feature size is typically lower than for EBL and is limited by the technique used to pattern the master and the diffusion of the inks during stamping. For many biological applications, practical feature sizes are in the range of tens to hundreds of micrometers (67, 68). Elastomeric polymer stamps can be used to microstructure large surface regions in parallel, as long as the patterns are not too complex. For example, μCP was used to pattern neuronal stem cells on poly-lactic-*co*-glycolic acid polymer (69). Neuronal stem cells are a particularly important target for patterning because of their potential role in neural repair and therapy (70).

Among the various soft lithographic methods, μCP is the most widely used soft lithographic microstructuring method. In most cases, the elastomeric polymer stamps are made of PDMS, although a range of different polymers can be used (71, 72). Among the more successful examples are perfluorinated elastomers, such as perfluoropolyether (PFPE). PFPE stamps were used for protein printing to guide cell adhesion, morphology, and spreading of primary human fibroblasts (73). Similarly, composite elastomeric materials containing acryloxy perfluoropolyether and PDMS can be used to prepare defined μCP geometries (74).

As mentioned above, SAMs of alkanethiolates constitute a widely used model for surface engineering. One of the reasons for the relative success of SAMs for biointerface applications is their excellent compatibility with conventional μCP techniques (75–77). Using the μCP technique, Lehnert and colleagues (68) reported a systematic study to relate cellular gross behavior to local surface geometries. Initially, a diverse set of microstructures was created by μCP of the hydrophobic alkanethiol octadecylmercaptan onto gold-coated glass slides. The square patterns varied from 0.3 μm to 3 μm, and the square-to-square distance was between 1 μm and 30 μm. Next, the surfaces were incubated with the ECM proteins fibronectin and vitronectin, which selectively adsorbed to the octadecylmercaptan patches. Cell culture with a wide range of different cells, such as mouse melanoma cells, B16 cells, rat liver cells, and NIH 3T3 fibroblasts, revealed that cells adhered and spread on patterned areas as small as 0.1 μm^2, as long as the spacing between features did not exceed 5 μm (**Figure 7**). In contrast, center-to-center distances above 30 μm did not support cell spreading when feature sizes were larger than 1 μm^2. This and similar studies are important, as they may guide the reconciliation of cellular microenvironments.

Compared with other patterning methods, soft lithographic microstructuring and, more specifically, the μCP technology are widely used because of the fact that they are simple, rapid, inexpensive, and compatible with variable ligation chemistries. It should be noted, however, that the use of SAMs under physiological conditions is limited to short-term studies because of the limited stability of SAMs owing to oxidation and desorption of alkanethiolates (78). A potentially more stable alternative with similar versatility is the CVD-based reactive coatings introduced above.

Soft lithography, and μCP in particular, may be readily combined with CVD substrates. This is advantageous in that CVD coatings are more stable than the SAMs of alkanethiols commonly used with μCP. In fact, μCP has been exploited in combination with CVD to generate spatioselective sensing platforms (79), to coimmobilize multiple biologically active ligands (80), and to spatio-selectively control cell adhesion and growth (81). For example, sufficiently thin (on the order of a few nanometers) CVD coatings were polymerized onto surface plasmon resonance (SPR) chips to facilitate the spatio-selective patterning of biotin-hydrazide moieties to create a sensing array (79). SPR may be used to probe the degree of biomolecular interactions between a ligand and an analyte for the purposes of drug discovery and diagnostics. After μCP, the array was backfilled with PEG hydrazide to make the background nonreactive, as PEG is known to be protein resistant. As a result, this CVD sensor was used to probe the spatio-selective interactions of a cascade of biomolecules.

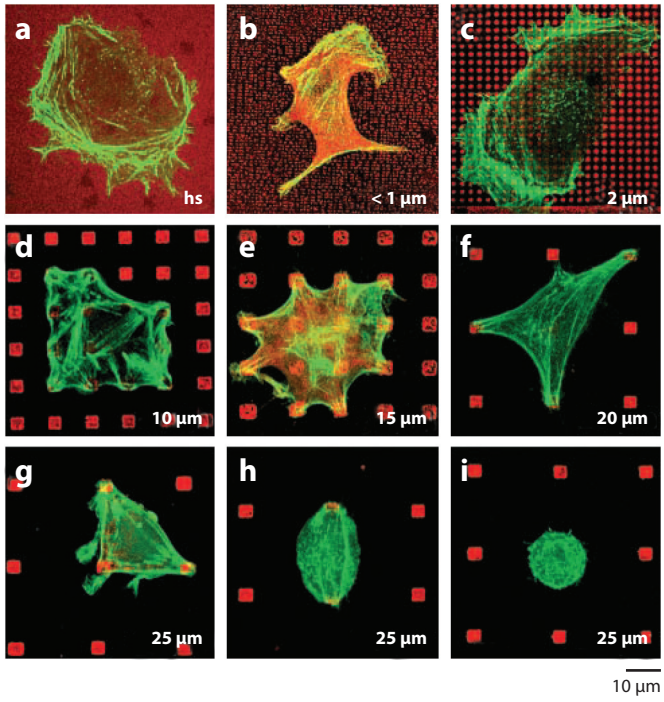


Figure 7

Cell spreading as a function of patterning. B16 cells were cultured on fibronectin substrata prepared with μCP and labeled for fibronectin (*red*) and actin (*green*). (*a*) On homogeneous substratum (hs), actin filaments are distributed throughout the cell periphery. (*b*,*c*) If the space between dots is 2 μm (*b*, 0.1 μm^2 squares 1 μm apart; *c*, 1 μm^2 squares 2 μm apart) cells spread as on a homogeneous substratum. (*d–i*) Cell growth on patterned substrata of 9 μm^2 dots with spacing as indicated in the right-hand corner. (*d–f*) With distances of 5–20 μm between dots, cells spread and the actin cytoskeleton forms stress fibers between adjacent dots. (*g–i*) At 25 μm spacing, spreading was limited and cells became triangular, ellipsoid, or round. Adapted with permission from Reference 68.

DIP PEN NANOLITHOGRAPHY

Dip pen nanolithography (DPN) uses an atomic force microscopy probe to directly write a solution with a high chemical affinity for a substrate. Microstructures are created by bringing ink molecules in contact with the substrate surface through the liquid meniscus surrounding the atomic force microscopy probe (82, 83). DPN is a rather complex interplay of several different factors, and the quality and specificity are influenced by humidity, tip geometry, writing speed, or contact time (84). Exact control of the ambient environment is critical for printing quality, and environmentally controlled cells or glove boxes are typically required. The utility of DPN is expanded when used with substrate-independent techniques, such as CVD, wherein DPN has been used to pattern a multitude of substrates, including Teflon, glass, and rubber (85). Furthermore, DPN allows for direct writing of two or more different ligands within the same microstructure. Multi-ligand micropatterns are difficult to obtain with soft lithographic methods, such as μCP. In addition, a plurality of different inks, including small molecules as well as biomacromolecules, can be processed by DPN (86–88). The deployment of multi-pin arrays has facilitated larger-area printing (47).

Successful multiplexing of multiple biomacromolecules via DPN was demonstrated by recent work aimed at creating lipid membrane microstructures for cell culture studies (89). The

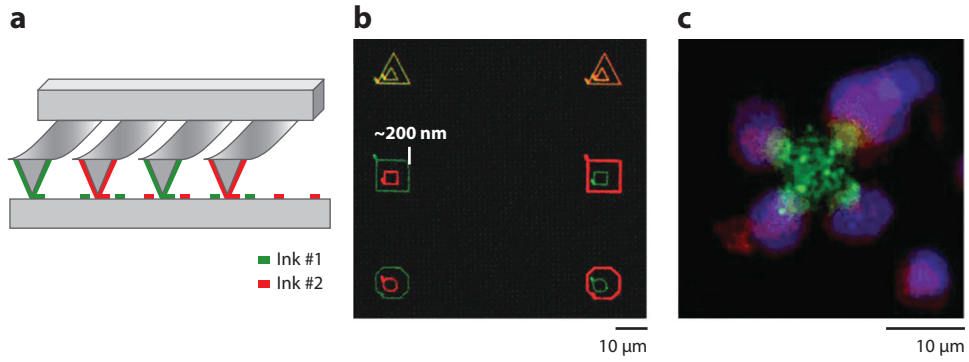


Figure 8

Lipid writing via dip pen nanolithography (DPN) and cellular response to said writing. (*a*) Schematic of DPN cantilever array used for writing. (*b*) Writing with two fluorophore labeled lipids (rhodamine/red and fluorescein/green). Yellow and orange triangles result from mixing the lipid inks in different concentrations. Fluorescence micrographs of T cells selectively adhered to and activated by functional proteins bound to phospholipid multilayer patterns via streptavidin. (*c*) A three-channel image of T cells adhering to the corners of lipid protein DPN patterns and activated by functional proteins. Green, red, and blue florescence represent the lipid pattern, cell activation, and Dapi nucleus staining, respectively. Adapted with permission from Reference 89.

multifunctional lipid membrane microstructures were then used for studying T-cell adhesion and activation. Interestingly, T cells selectively adhered to the rounded perimeters of the lipid membrane microstructures (**Figure 8**). Biointerface platforms, such as the ones described in this work, may spark exploration of a multitude of biological interactions with potential technological applications in diagnostics, drug discovery, or cell-based therapeutics.

As is true for other microstructuring methods, DPN has also been evaluated for the potential to guide stem cells (90). For example, DPN-based arrays of thiolates with various chemical functionalities were prepared on a gold substrate (90). Some of the thiolate end groups included $-COOH$, $-NH_2$, $-CH_3$, and $-OH$. DPN microstructuring allowed for defined spacings between 140 nm and 1,000 nm. Finally, MSCs were cultured on the substrates, and the authors observed that both the feature density and chemistry influence the formation of focal adhesion contacts. Specifically, increasing the distance between features above 280 nm on OH-terminated SAMs decreased the number of cell focal contacts and induced differentiation of MSCs toward an osteogenic lineage.

COLLOIDAL LITHOGRAPHY

This interfacial microstructuring method takes advantage of two-dimensional arrays of colloidal particles that are used as shadow masks (91). This emerging technology is fast, can cover large surface areas, is inexpensive, and can be applied to a wide range of substrate choices. Potential limitations stem from the fact that particles are used as shadow masks: First, the particles require self-assembly of a monolayer, which can be tedious and is often prone to defects. Moreover, micropattern geometry and accessible feature size are limited, because they are defined by the size and geometry of the particles. Also, the technology is very sensitive to particle heterogeneity or polydispersity. Typically, the micropatterns that can be generated with colloidal lithography are limited to simple features, such as triangles or spheres (92, 93).

Colloidal lithography has been used to study the interaction of breast cancer cells with microstructured ECM proteins (94). ECM patterns with sizes of 100 nm, 200 nm, 500 nm, and

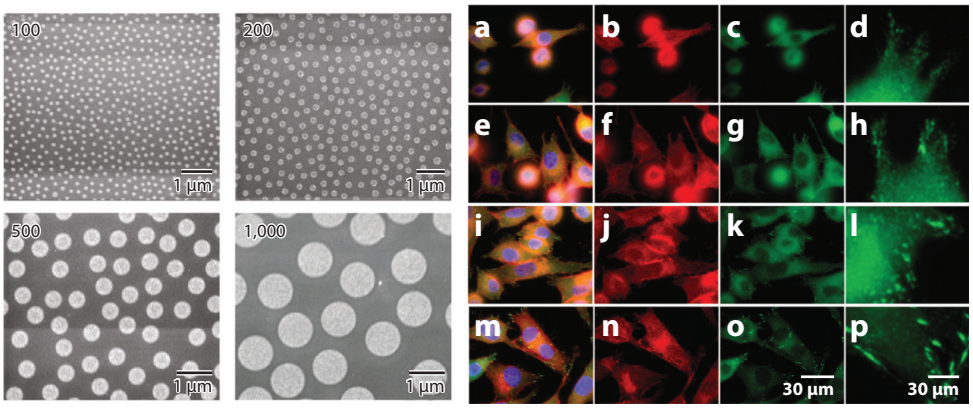


Figure 9

SEM images of 100–1,000-nm gold holes (bright) in SiO2 film (*left*). Fluorescence microscopy of breast cancer cells with red staining of actin, green staining of vinculin, and blue DAPI staining of the nucleus on patterned substrates of (*right*) (*a–c*) 200 nm, (*e–g*) 500 nm, and (*i–k*) 1,000 nm (*m–o*) homogeneous control. Adapted with permission from Reference 94.

1,000 nm were generated using colloidal lithography. After incubation of the substrates with the breast cancer cells, cell adhesion, spreading, and stress fiber formation were assessed. The authors identified the size of the protein patches as an important factor influencing cell adhesion and spreading (**Figure 9**). This work demonstrates the potential of colloidal lithography for ECM organization studies and cell surface signaling studies.

DEFINED CULTURE SYSTEMS FOR PLURIPOTENT STEM CELLS

The in vitro environment plays a critical role in the adhesion, proliferation, and gene expression of biological cells. An area where this is particularly evident is the in vitro expansion of pluripotent stem cells. This area of research provides major opportunities to biomedical researchers because of the cells' potential clinical utility and the technological challenges associated with their culture.

Firstly, the potential impact of pluripotent cells, such as human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs), is enormous. Because these cells have the capacity for self-renewal, they can in principle be expanded to therapeutically and technologically critical numbers. Their pluripotency allows them to differentiate into all cells of the three different germ layers and the trophoectoderm. This makes them potential cell sources for regenerative therapies, novel clinical studies with disease-specific stem cells, and pharmaceutical in vitro screening of future drug candidates. Although these aspects are equally true for hESCs and iPSCs, there are profound differences in their origins, which will impact their clinical utility in the future. hESC lines must be derived from embryos, which makes their use controversial and can elicit immune responses when transferred to an unrelated patient. However, derivation of disease-specific stem cell lines may be important for elucidating novel pathways and clinical targets. In contrast, iPSCs are derived from adult cells, such as fibroblasts, which are typically accessible directly from a patient. The main advantage of patient-derived stem cells is the fact that cell therapies do not require immunomodulation. To date, the derivation of iPSCs requires genetic reprogramming, which raises substantial safety concerns.

Because pluripotent stem cells have the ability to spontaneously differentiate into many other cells, they are particularly sensitive to the local cellular microenvironment. As a consequence,

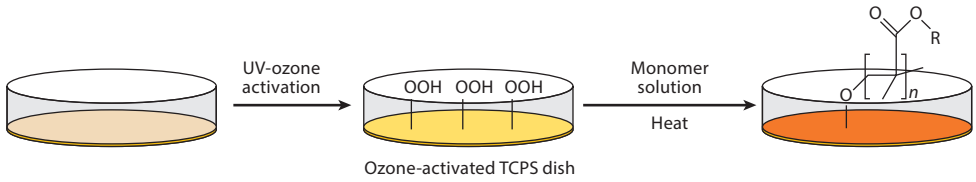
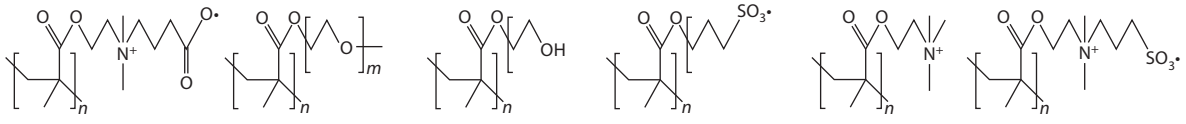


Figure 10

Schematic depicting the graft-polymerization process used to fabricate the polymer coatings. UV ozone was used to activate the tissue culture polystyrene dishes, and then methacrylate-based monomers were subsequently polymerized on the surface. Adapted with permission from Reference 104.

hESCs and iPSCs cannot simply be expanded in petri dishes or bioreactors, as is the case for mature cells. Pluripotent stem cells undergo an undirected differentiation into so-called embryoid bodies under these conditions. Expansion of pluripotent stem cells without spontaneous differentiation has been successfully achieved on human and murine feeder cells. In this case, the stem cells are cultured directly on a layer of support cells, such as murine fibroblasts. However, stem cell populations expanded on feeder cells are heterogeneous; prone to batch-to-batch variability; and exposed to a completely undefined microenvironment that, in the case of murine feeder cells, contains xenogeneic contaminants of unknown composition. For all these reasons, feeder cultures are not ideal for expansion of clinically relevant cell populations. A potential alternative has been identified in the form of Matrigel™. Matrigel is an undefined gelatinous protein mixture isolated from mouse carcinoma cells and is widely used for hPSC culture. It is simpler to use than feeder cultures but is still plagued with the same shortcomings, such as undefined culture environments leading to heterogeneous cell populations and xenogeneic contaminations. This creates a clear opportunity for well-defined and xenofree synthetic materials that can support the undifferentiated expansion of pluripotent stem cells similar to feeder cultures or Matrigel, but paired with the added value of simplified use and defined culture systems typically known from petri dishes. Recently, human



	PCBMA	PPGEMA	PHEMA	PSPMA	PMETAC	PMEDSAH
Contact angle	71.6 ± 4.8	63.3 ± 3.1	56.0 ± 3.7	50.2 ± 4.1	40.5 ± 5.7	17.1 ± 1.1
Reduced elastic modulus (GPa)	3.3 ± 0.1	3.9 ± 0.1	3.0 ± 0.5	3.0 ± .01	3.3 ± 0.1	2.5 ± 0.1
Cell-aggregate adhesion (%)	0	5 ± 1	12 ± 1	14 ± 2	8 ± 1	15 ± 1
Number of passages	0	1	2	2	2	25

Figure 11

Long-term culture of H9 human embryonic stem cells (hESCs) on methacrylate-derivative coatings with mouse embryonic fibroblast–conditioned media. Table provides information about substrate properties [contact angle, reduced elastic modulus (GPa) (mean ± SD)] and cell behavior [initial hESC aggregate adhesion (mean ± SEM) and number of passages achieved] on each polymer coating. Adapted with permission from Reference 104.

recombinant proteins of the ECM, such as the laminin isoforms −111, −332, 511, vitronectin, and E-cadherin, have been employed as feeder-free substrates in long-term maintenance studies of hESCs (95–97). Similarly, ECM peptides have been successfully incorporated into synthetic hydrogels. In particular, Synthemax^{TM} (Corning) is an acrylate polymer with covalently immobilized amine-containing peptides on the surface (98). Alternatively, the heparin-binding peptide GKKQRFRHRNRKG was conjugated to SAMs and showed good results in hESC culture (99). These proteins and peptides are indicative of a novel trend from feeder-cell-dependent and non-defined culture conditions to feeder-free and fully defined microenvironments (100). However, purified human recombinant proteins and peptides are labor and cost intensive and significantly limit the clinical potential of these culture dishes.

In an early attempt to replace the poorly defined and extremely expensive biological matrices, fully synthetic coatings have been studied as potential feeder cell replacements (101–103). Recent work has highlighted the potential utility of certain zwitterionic hydrogel films as a platform for stem cell culture (104, 105). Fully synthetic substrates can be generated using a simple, reproducible, and well-documented (106) surface-initiated graft polymerization process (**Figure 10**).

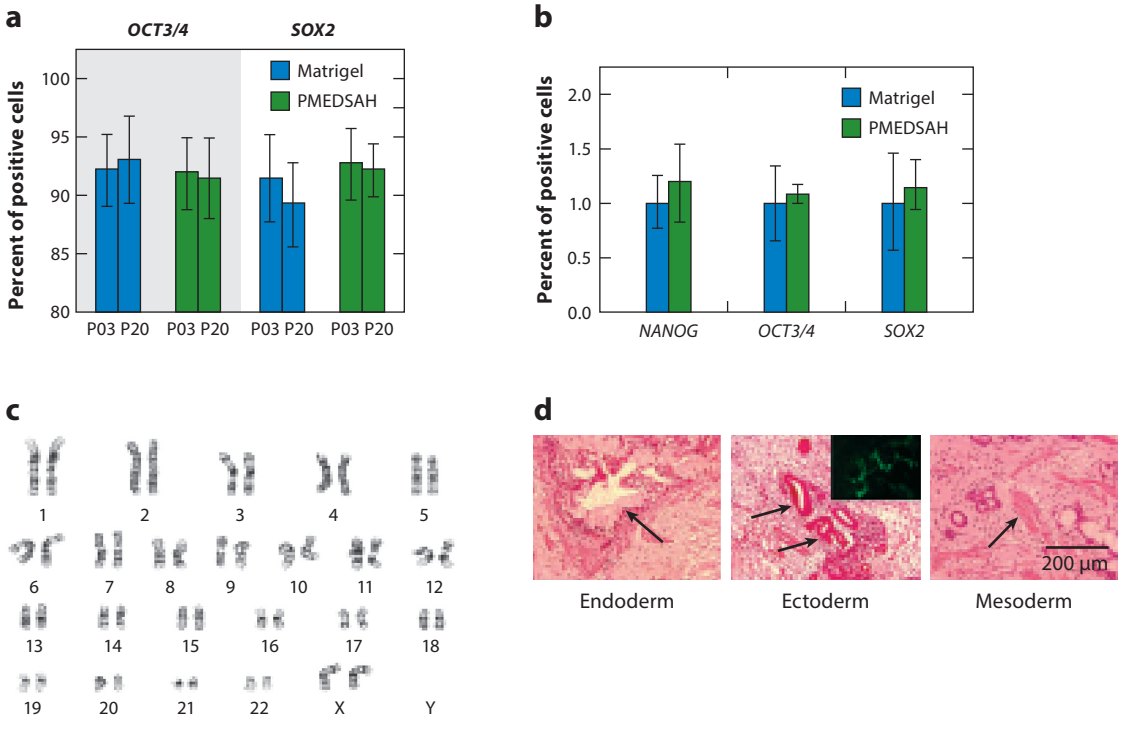


Figure 12

Cellular characterization of human embryonic stem cells (hESCs) cultured on PMEDSAH substrates in MEF-CM. (*a*) Percentage (mean ± SEM) of hESCs expressing OCT3/4 and SOX2 at passages 3 (P03) and 20 (P20). (*b*) Relative transcript levels of *NANOG*, *OCT3/4*, and *SOX2* from hESCs cultured on PMEDSAH and Matrigel^{TM}. (*c*,*d*) After 25 passages, hESCs cultured on PMEDSAH (*c*) maintained a normal karyotype and (*d*) retained pluripotency as demonstrated by teratoma formation in immunosuppressed mice. Hematoxylin- and eosin-stained paraffin sections indicating endoderm (goblet-like cells at arrow), ectoderm (neuroepithelial aggregates at arrow, and cells expressing neuron-restricted protein β-III tubulin in inset), and mesodermal derivatives (cartilage, connective tissue, and muscle at arrow). Adapted with permission from Reference 104.

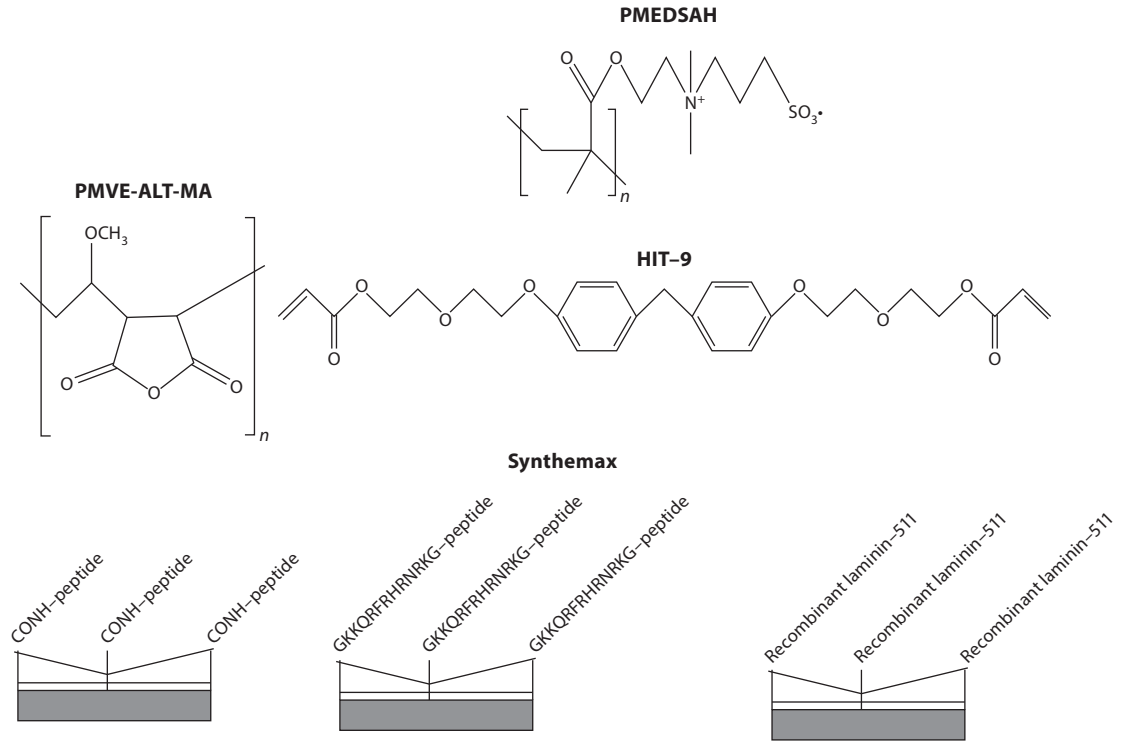


Figure 13

Synthetic stem cell culture materials used for long-term maintenance of human pluripotent stem cells. Adapted with permission from Reference 115.

Several methacrylate derivatives have been assessed in cell screening with hESCs, such as poly[carboxybetaine methacrylate], poly([2-(methacryloyloxy)ethyl]trimethylammonium chloride), poly[poly(ethylene glycol) methyl ether methacrylate], poly[2-hydroxyethyl methacrylate], poly[3-sulfopropyl methacrylate], and, most importantly, poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH) (**Figure 11**) (104).

Of the aforementioned polymers, only the PMEDSAH polymer was able to support undifferentiated hESCs (BG01 and H9 cell lines) over more than 25 passages. The hESCs retained normal gene expression, karyotype, and embryoid body formation (**Figure 12**). Results from the zwitterionic substrates were compared with those from Matrigel.

Recently, a variety of fully synthetic substrates (107–114) have emerged, and several have demonstrated their potential for large-scale expansion of clinical-grade hESCs (**Figure 13**). Synthetic substrates generally have defined chemical composition, stable storage, reproducible synthesis, cost-effectiveness, scalability, and improved compatibility with standard sterilization techniques. **Table 3** provides a comparison of the synthetic substrates described in this work, relative to one another and to Matrigel, the current gold standard for stem cell culture in defined media conditions.

These initial studies have been focused mainly on the maintenance of pluripotent cells in their undifferentiated state during long-term culture. Because the clinical utility of hPSCs comes from their ability to provide access to specific adult cells, future work will be increasingly directed toward differentiation into specific cell lineages, such as nerves, cartilage, or bone, in fully defined

Table 2 Comparison of the various patterning techniques employed to investigate cell behavior (43)

Technique	Length scale (μm)	Advantages	Limitations	Biomolecular patterning approach
Electron beam lithography	0.005 (44)	High resolution; compatible with standard microfabrication techniques, which allows its incorporation into biochips and biosensors	Relatively expensive; slow patterning speed	Indirect
Photolithography	0.1 (45)	Varied patterns over large areas; compatible with numerous substrates	Relatively expensive; high resolution	Indirect
Soft lithography	0.1 (46)	Simple implementation and patterning of large areas; relatively inexpensive	Cannot simultaneously print multiple inks; concerns with ink diffusion	Direct
Dip pen nanolithography	0.015 (47)	Writing can occur within patterns to create complex surface architectures; compatible with broad range of inks and can be used for high-throughput applications	Printing quality dependent on a myriad of environmental and system parameters	Either
Colloidal lithography	0.01 (48)	Relatively inexpensive and simple; rapid and patterns large surface areas; capable of 3D patterning	Reduced user-defined control of size and geometries as these dependent on particle physics	Indirect
Microfluidic patterning	0.1 (49)	Relatively inexpensive; rapid, dynamic systems; multiple ligands and cells can be patterned and subcellular processes studied	Channel geometry limits pattern diversity	Direct

culture environments. This will constitute the next milestone and will underpin the potential of pluripotent stem cells for various medical applications in tissue engineering and regenerative medicine. Already, a small number of important studies have attempted directed differentiation of hPSCs in defined microenvironments (116, 117). For example, hMSCs were derived from hESCs and were loaded into a PEG-based polymer modified with ECM proteins (118). The stem cells were successfully expanded in vitro for six weeks to induce chondrogenic or osteogenic differentiation. Others have shown that hMSCs can be derived from iPSCs (105). In this case, the iPSCs were directly derived by reprogramming a donor's own fibroblasts. An important aspect of the latter approach is that every single cell-handling and culture step can be conducted on fully defined, synthetic substrates. These and other systematic studies on the role of stem cell differentiation and sourcing under fully defined culture conditions will be critical in developing clinically relevant cell populations (119, 120).

CONCLUSIONS

A host of methods and materials are available to scientists and engineers for generating biointerfaces that mimic the complex cellular microenvironment. These tools have been exploited

Table 3 Comparison of synthetic substrates and Matrigel™ for long-term human pluripotent stem cell (hPSC) culture (115)

Substrate type	Reusable	Passage # tested	Prep for cell culture use	Can be sterilized via large bath methods?	Relative cost	Fabrication	Cell type
PMEDSAH	Yes	25	Used as is	Yes; E-beam and gamma radiation	Inexpensive	Polymeric Grattinq	hESC (several types)
Synthemax™ (Corninq)	No (b/c peptide)	≥10	Used as is	Gamma radiation; subject to degradation after gamma exposure (b/c peptide)	Expensive (b/c of peptides)	Photopolymerization and chemical conjugation of peptide via EDC/NHS	hESC (several types)
GKKQRFRH-RNRKG	No (b/c peptide)	17	Used as is	Subject to degradation after gamma exposure (b/c peptide)	Expensive (b/c of peptides)	Physisorption	hiPSC and hESC
PMVE-alt-MA	Yes	5	Used as is	UVC germicidal radiation	Inexpensive	Free radical polymerization	hiPSC and hESC
hit 9	No (b/c of need of protein adsorption)	≥5	Requires preadsorption of extracellular matrix protein vitronectin	Unknown	Inexpensive	Photopolymerization	hiPSC and hESC
Human recombinant laminin-511	No (b/c protein)	≥20	Used as is	Subject to degradation after gamma exposure (b/c peptide)	Expensive	Physisorption	hiPSC and hESC
Matrigel™	No	≥20	Yes	No	Expensive	Cell feeder layers	hiPSC, hESC, hMSCs, etc

to augment the chemical and physical properties of the cellular microenvironment on the micro- and nanoscale, and the advantages and limitations of some of these approaches have been explored. As technology improves, it is expected that new and enhanced techniques will be realized. Of the approaches highlighted herein, CVD is a highly attractive technique, as it is substrate independent and is compatible with many of the patterning/topographic augmentation strategies highlighted in this work. As stem cells represent a promising platform for tissue engineering and regenerative medicine, the importance of engineered biointerfaces for stem cells was emphasized in the context of generating cells in an environment that lends itself to

clinical adoption. Though significant progress has been made, there is still a need to explore these complex microenvironments, particularly in three-dimensional and in chemically defined microenvironments, to facilitate clinical translation.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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