Published in final edited form as:

Soft Matter. 2011 October 21; 7(20): 9561–9571. doi:10.1039/C1SM05596H.

Chemically well-defined self-assembled monolayers for cell culture: toward mimicking the natural ECM

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Abstract

The extracellular matrix (ECM) is a network of biological macromolecules that surrounds cells within tissues. In addition to serving as a physical support, the ECM actively influences cell behavior by providing sites for cell adhesion, establishing soluble factor gradients, and forming interfaces between different cell types within a tissue. Thus, elucidating the influence of ECM-derived biomolecules on cell behavior is an important aspect of cell biology. Self-assembled monolayers (SAMs) have emerged as promising tools to mimic the ECM as they provide chemically well-defined substrates that can be precisely tailored for specific cell culture applications, and their application in this regard is the focus of this review. In particular, this review will describe various approaches to prepare SAM-based culture substrates *via* non-specific adsorption, covalent immobilization, or non-covalent sequestering of ECM-derived biomolecules. Additionally, this review will highlight SAMs that present ECM-derived biomolecules to cells to probe the role of these molecules in cell-ECM interactions, including cell attachment, spreading and ‘outside-in’ signaling *via* focal adhesion complex formation. Finally, this review will introduce SAMs that can present or sequester soluble signaling molecules, such as growth factors, to study the influence of localized soluble factor activity on cell behavior. Together, these examples demonstrate that the chemical specificity and variability afforded by SAMs can provide robust, well-defined substrates for cell culture that can simplify experimental design and analysis by eliminating many of the confounding factors associated with traditional culture substrates.

1. Introduction

The extracellular matrix (ECM) is a complex network of self-assembled biological macromolecules, such as proteins, glycoproteins, and proteoglycans, that provides

mechanical support, presents sites for cell anchorage, establishes soluble factor gradients, and forms interfaces between distinct cell types within connective tissues.¹ The macromolecular composition of the ECM consists primarily of collagens, a large family of structural proteins that are ubiquitous within vertebrate ECMs.² Beyond the collagenous component, the vertebrate ECM can be highly variable and is often specific to a given tissue type. A few specific examples help to illustrate the diversity of vertebrate ECMs. The ECM of vertebrate bone can generally be divided into two phases: 1) an organic 'osteoid' phase comprised of collagens, proteoglycans (*e.g.* heparan sulfate), proteins that regulate mineral nucleation and growth *via* mineral-binding domains (*e.g.* osteocalcin, osteopontin, and bone sialoprotein), and structural/ adhesive proteins (*e.g.* fibronectin); and 2) an inorganic hydroxyapatite phase.³ Similarly, the ECMs of mechanically compliant tissues (*e.g.* cardiovascular tissues, respiratory tissues, and the bladder) are primarily comprised of collagenous structural proteins; however, the ECM of these tissues also contains a highly elastic protein elastin, which introduces the mechanical compliance and elasticity required for proper physiological function.⁴ The composition of the ECM of the adult brain, on the other hand, is significantly different from the ECM of most other connective tissue types. In particular, the density of collagens and other structural proteins is quite low, and instead, hyaluronic acid-binding proteoglycans of the lectican family, including versican, neurocan, and brevican, as well as hyaluronic acid and tenascins, are the primary components of the brain ECM.⁵ In light of these diverse natural ECM compositions, recent research efforts have focused on characterizing the influence of various ECM macromolecules in connective tissue development and homeostasis. For example, significant progress has been made toward understanding how cell adhesion and proliferation are modulated by specific macromolecules present within the ECM of a given tissue type.⁶ Moreover, noteworthy efforts have begun to elucidate the role of the ECM in regulating stem cell self-renewal and differentiation.⁷ Cell culture substrates play a critical role in studying cell-ECM interactions, and emerging approaches are progressing toward well-defined and adaptable presentation of ECM components to cells. This review will highlight chemically well-defined cell culture substrates, with an emphasis on studies that use novel substrates to characterize cell response to ECM-derived biomolecules. In addition, this review will introduce a new class of substrates inspired by native non-covalent ECM assembly mechanisms that sequester biomolecules to modulate cell behavior.

2. Biomaterials as cell culture substrates

Synthetic polymeric substrates (*e.g.* polystyrene) that have been chemically treated to allow non-specific biomolecule adsorption are routinely used to probe the influence of ECM-derived biomolecules on cell behavior.⁸ For example, to study the role of fibronectin in cell adhesion, a polystyrene substrate can be bathed in a solution containing fibronectin, and then cells can be seeded onto the protein-coated substrate. To date, this approach has been successfully applied to study the influence of ECM-derived biomolecules on cell behavior. However, the importance of finer details, such as biomolecule orientation or density, are difficult to study using this approach due to the random nature of non-specific biomolecule adsorption onto a substrate.⁹ In light of this limitation, immobilizing biomolecules onto synthetic materials *via* specific covalent and non-covalent mechanisms has recently emerged

as a powerful tool to probe in greater detail the influence of ECM-derived biomolecules on cell behavior. For example, strategies have been developed to immobilize biomolecules on highly hydrated synthetic polymer matrices, termed 'hydrogels'. The properties that make hydrogels attractive substrates for cell culture include their physical similarity to the native ECM (*i.e.* highly hydrated, viscoelastic), their synthetic adaptability, their ease of formation (including self assembly of fibrillar matrices), and their tendency to limit non-specific interactions with proteins and cells. Hydrogels have also enabled emerging cell culture studies in three-dimensional microenvironments, in which cell-material interactions are fundamentally distinct from more common 2-dimensional cell culture substrates.¹⁰ Studies that use hydrogel matrices to characterize cell response to ECM-derived biomolecules have been extensively reviewed elsewhere by us and others,¹¹ and will not be a topic of this review. In another approach to study cell-material interactions, we and others have used self-assembled monolayers (SAMs) of alkanethiolates on gold as chemically well-defined cell culture substrates. SAMs are particularly advantageous for well-defined, hypothesis-driven cell culture experiments, as they provide a well-defined molecular monolayer that can be adapted to include a wide range of chemical moieties, including bio-inert polymer chains, cell-interactive peptides, ECM proteins, and molecular sequestering ligands. SAMs are also amenable to spatiotemporal patterning, which has led to important advances in our understanding of cell spreading, cell geometry, cell-cell interactions, and cell migration. The use of SAMs as tools to understand the role of ECM biomolecules on cell behavior will be the focus of this review.

2.1 Self-assembled monolayers of alkanethiolates on gold

Self-assembly is a process wherein molecules interact *via* specific and reversible non-covalent interactions to mediate the formation of higher-ordered structures. Self-assembly is commonly observed in biological and non-biological contexts. For example, the membrane surrounding mammalian cells is formed by self-assembly of phospholipids and proteins.¹² Additionally, the extracellular matrix that surrounds cells is formed by the self-assembly of collagenous proteins with proteoglycans¹³ and other proteins.¹⁴ Self-assembly can also take place in a non-biological context, resulting in well-ordered, synthetic materials. In 1983, Nuzzo and Alara observed that thiol groups form strong and specific non-covalent coordination bonds with gold that, in turn, result in the formation of a monolayer of thiolated molecules on gold-coated materials.¹⁵ Subsequently, thiol-terminated alkyl chains (herein termed 'alkanethiolates') were observed to self-assemble into close-packed monolayers on gold substrates through the concerted influence of sulfur-gold coordination bonds and van der Waals interactions between alkyl chains¹⁶ (Fig. 1). Since these early observations, SAMs of alkanethiolates on gold have emerged in widespread applications requiring chemically well-defined substrates, such as electrochemical biosensors, physical chemistry, bioanalytical chemistry, and bio-organic chemistry, and their use in these applications has been extensively reviewed elsewhere.¹⁷

2.2 SAMs on gold as chemically well-defined cell culture substrates

The rapid formation of stable monolayers in the absence of external stimuli¹⁸ and the reliance on gold, a relatively inert metal that resists oxidation and atmospheric contamination,¹⁹ makes SAMs of alkanethiolates on gold a promising material to engineer

cell culture substrates. A particular advantage of SAMs is their ability to present ECM-derived biomolecules in a chemically well-defined environment, which allows investigators to address specific hypotheses related to cell-biomolecule interactions. The following sections will highlight the development of SAMs as cell culture substrates and the use of SAM-based cell culture substrates to study the relationship between ECM-derived biomolecules and cell function.

2.2.1 Non-specific protein adsorption onto SAM-based cell culture substrates

—Similar to the standard tissue culture treated polystyrene substrates described above, SAMs are routinely used as cell culture substrates to study the influence of adsorbed proteins on cell function. A unique distinction of SAMs when compared to traditional culture substrates, however, is that alkanethiolates terminated with a wide variety of chemical functionalities can be readily synthesized, and these functionalized alkanethiolates efficiently pack into SAMs on gold.²⁰ This chemical variability has provided a unique tool to probe the influence of biomaterial surface chemistry on protein adsorption and, in turn, cell interaction with adsorbed proteins (Fig. 2). For example, SAMs have been used to characterize the influence of biomaterial surface chemistry on cell adhesion (*e.g.* human umbilical vein²¹ and bovine aortic endothelial cells,²² mesenchymal stem cells,²³ platelets,²⁴ osteoblasts²⁵ and osteoblast-like cells,²⁶ neutrophils,²⁷ corneal epithelial cells,²⁸ leukocytes,²⁹ adipostromal cells,³⁰ and fibroblasts³¹), proliferation (*e.g.* fibroblasts,³² osteoblast-like cells,³³ keratinocytes,³⁴ myoblasts,³⁵ adipostromal cells,³⁰ and neurons³⁶), and differentiation (*e.g.* myoblasts,³⁵ mesenchymal stem cells,³⁷ osteoblasts³⁸ and adipostromal cells³⁰). In addition, SAMs have been used to study surface chemistry features that influence embryonic stem cell expansion. For example, Wu and co-workers showed that surface hydrophobicity influences the size of murine embryoid bodies and, in turn, influences cell viability, proliferation, and maintenance of pluripotent phenotype.³⁹ SAMs comprised of alkanethiolates terminated with chemically distinct functional groups have also been used to study the influence of surface chemistry on neuron physiology. Shimizu and co-workers demonstrated that amine-terminated SAMs enhance neurite outgrowth,⁴⁰ while Sweedler and co-workers demonstrated that surface chemistry influences the dynamic properties of neuronal action potentials.³⁶ Together, these studies underscore the importance of surface chemistry as a key determinant of cell interaction with non-specifically adsorbed biomolecules, a feature that may have been challenging to identify using traditional polystyrene substrates due to the inherent complexity associated with modifying the surface chemistry of polymeric materials.

“Bio-inert” SAMs: Poly(ethylene glycol) is a hydrophilic, synthetic polymer that demonstrates limited interaction with proteins.⁴¹ In 1991, Whitesides and co-workers demonstrated that alkanethiolates terminated with oligo(ethylene glycol) (OEG) moieties form SAMs on gold and, in turn, confer the monolayer with resistance to non-specific protein adsorption.⁴² The observation that surface chemistry influences biomolecule adsorption and, in turn, cell interaction with adsorbed biomolecules suggested that spatially patterning regions with different surface chemistries may provide a way to spatially pattern cell interaction with the substrate. To test this hypothesis, Whitesides and co-workers developed a micro-contact printing method to spatially pattern islands of alkanethiolates that

mediate non-specific protein adsorption within a background of cell- and protein-resistant OEG alkanethiolates. In an initial demonstration of patterned SAMs as cell culture substrates, Ingber, Whitesides and co-workers prepared SAMs with different sized fibronectin-coated islands and demonstrated that adhesive island size and, in turn, extent of cell spreading dictated human and bovine aortic endothelial cell apoptosis or growth.⁴³ Since this initial demonstration, micro-contact printed SAMs with neighboring cell adhesive and non-adhesive domains have been used extensively to study the influence of cell shape on cell behavior, especially in the context of adult stem cell differentiation. For example, Chen and co-workers demonstrated that mesenchymal stem cells preferentially differentiated into osteoblasts when allowed to spread. This effect was attributed to increased actin-myosin tension and, in turn, up-regulated RhoA-ROCK signaling. However, confining hMSCs to a rounded morphology, which decreased actin-myosin tension and down-regulated RhoA-ROCK signaling, directed the cells towards an adipogenic phenotype.^{37b} More recently, Mrksich and coworkers demonstrated that cells confined to geometric shapes that favor actin-myosin contractility direct cells towards an osteogenic phenotype, while shapes that decrease actin-myosin contractility direct cells towards an adipogenic phenotype, regardless of the extent of cell spreading within the patterned shape.^{37c} Taken together, these studies establish that patterning of alkanethiolates by micro-contact printing - a method that is not amenable to traditional polystyrene culture substrates - is a useful strategy to probe the role of actin-myosin contractility in diverse cell processes and discover key intracellular signaling processes involved in adhesion-mediated modulation of cell behavior.

2.2.2 SAM-based cell culture substrates formed from peptide-terminated

alkanethiolates—In addition to providing a means to pattern cell adhesive and cell repulsive islands on a single SAM, Whitesides and co-workers demonstrated that SAMs resistant to non-specific protein adsorption could be engineered to promote specific cell or protein interactions through chemical modification of the alkanethiol. In the initial demonstration of this approach, they chemically modified otherwise bio-inert OEG alkanethiolates to present the ECM-derived cell adhesion epitope Arg-Gly-Asp (RGD). SAMs formed from this alkanethiol allowed for integrin-mediated endothelial cell adhesion onto the monolayer in an RGD concentration-dependent manner.⁴⁴ Since this early demonstration, numerous efforts have relied on SAMs presenting ECM-derived biomolecules in an otherwise bio-inert background to study the specific influence of a given biomolecule on cell behavior. For example, Mrksich and co-workers used SAMs comprised of an OEG-terminated alkanethiolate of varying number of OEG repeat units and an RGD-terminated alkanethiolate to characterize the influence of the surrounding chemical environment on cell attachment to RGD.⁴⁵ Interestingly, cell attachment and spreading were dependent on RGD density when hexa(ethylene glycol)-terminated alkanethiolates were used, however, no correlation was observed when tri(ethylene glyco)-terminated alkanethiolates were used. More recently, Spatz and co-workers used SAMs comprised of RGD-terminated alkanethiolates on arrays of gold dots to characterize the relationship between RGD intermolecular distance and cell adhesion, spreading, polarization, and focal adhesion complex formation.⁴⁶ Additionally, Kiessling and co-workers identified peptides that bind to pluripotent stem cells using surface arrays⁴⁷ or phage-display,⁴⁸ then used SAMs formed from alkanethiolates terminated with these peptides to identify optimal

culture substrates for pluripotent stem cell expansion. Importantly, these examples demonstrate that peptide-decorated OEG SAMs can be used to characterize the influence of a peptide on cell function, and the well-defined SAM substrates eliminate confounding factors introduced *via* random, non-specific protein adsorption onto standard cell culture substrates.

2.2.3 Covalent immobilization of biomolecules onto SAM-based cell culture substrates—

Biomolecules can be covalently linked to otherwise bio-inert SAMs terminated with reactive functional groups (*e.g.* carboxylate,⁴⁹ maleimide⁵⁰ and aldehyde⁵¹), and this strategy is commonly used to immobilize ECM-derived biomolecules for cell culture applications. For example, Bartic and co-workers demonstrated that covalent immobilization of the laminin-derived peptide CSRARKQAA-SIKVAVSADR (also known as PA22-2), which has been shown to bind to $\alpha_6\beta_1$ integrin receptors and mediate cell adhesion and neurite outgrowth,⁵² is sufficient to promote neuronal cell attachment and dendrite outgrowth on SAMs.⁵³ Gopferich and co-workers used RGD covalently immobilized on a SAM in a quartz crystal microbalance designed for label-free characterization of cell adhesion dynamics,⁵⁴ while Mrksich and co-workers used RGD SAMs to study cell migration in response to soluble RGD.⁵⁵ In addition to immobilized peptides, gradients of covalently immobilized fibronectin, vascular endothelial growth factor, or both, have been used to characterize endothelial cell migration in response to these proteins.⁵⁶ Importantly, growth factors covalently immobilized onto SAMs often retain some biological activity, as demonstrated by the phosphorylation of epidermal growth factor receptors on SAMs presenting epidermal growth factor⁵⁷ or fibroblast growth factor-receptor mediated adhesion of cells onto SAMs presenting fibroblast growth factor-2.⁵⁸ These results suggest that growth factors can be covalently immobilized onto SAMs to mimic some aspects of the localized growth factor presentation that is observed within native extracellular matrices.

The immobilization strategies described above rely on functional groups that are common to biomolecules to mediate protein immobilization, most notably primary amines, carboxylates, and thiols. Although this strategy can simplify the immobilization process, it may also complicate the process by limiting the control over biomolecule orientation on the SAM, as common reactive functional groups are typically present in multiple locations on the biomolecule of interest. To address this limitation, numerous examples of chemoselective reactions that do not involve native protein functional groups have been developed to immobilize biomolecules on SAMs. For example, cyclopentadiene-bearing peptides can be immobilized to SAMs prepared from alkanethiolates terminated with a hydroquinone *via* the Diels–Alder mechanism.⁵⁹ Comparison of cell adhesion to RGD and Pro-His-Ser-Arg-Asn (PHSRN) conjugated to reactive groups presented by a SAM *versus* cell adhesion onto monolayers formed from alkanethiolates already terminated with RGD or PHSRN demonstrated that cell adhesion onto SAMs was mediated by specific cell-peptide interactions, regardless of the method used to create the peptide-linked SAM. The Diels–Alder mechanism was also used to immobilize low-affinity linear RGD peptides and high-affinity cyclic RGD peptides to correlate integrin-ligand affinity to formation of focal adhesion complexes by adherent cells.⁶⁰

To provide greater control over biomolecule orientation on SAMs, Mrksich and co-workers developed an approach that relies on formation of a covalent bond between an enzyme and a 'suicide ligand'. In particular, they prepared a fusion of the 10th FNIII domain of fibronectin and cutinase, an enzyme that forms a covalent bond upon reaction with a phosphonate ligand,⁶¹ and demonstrated that the cutinase-fibronectin fusion mediates specific adhesion of cells onto phosphonate-terminated SAMs.⁶² More recently, Mrksich and co-workers used this same methodology to characterize, in a well-defined context, the interaction of cell surface integrin receptors with the 9th and 10th FNIII domains of fibronectin.⁶³ Together, these examples show that one can use recombinant DNA technology to create fusions of a surface reactive protein and a cell-interactive protein, which enables controlled protein orientation on SAM-based cell culture substrates.

Recently, we used the copper(I)-catalyzed azide-alkyne cyclo-addition (CuAAC), a type of 'click' reaction, to immobilize RGD on SAMs and probe the influence of this ECM-derived ligand on human mesenchymal stem cell behavior⁶⁴ (Fig. 3). Our results demonstrated that RGD density on the SAM after the CuAAC reaction is directly dependent on the azide surface density on the SAM. In turn, we relied on this control over immobilized peptide density to characterize the influence of RGD surface density on human mesenchymal stem cell adhesion. Our results demonstrated that RGD surface density influenced hMSC attachment, spreading, and focal adhesion complex density. In particular, SAMs presenting a low density of RGD demonstrated few adherent stem cells that adopted a rounded morphology, while SAMs presenting a high density of RGD demonstrated numerous well-spread stem cells. Interestingly, the extent of cell spreading observed on these high-density RGD SAMs is similar to that observed when cells are cultured on tissue culture treated polystyrene in the presence of non-specifically adsorbed proteins. Together, these examples establish covalent immobilization of biologically-active peptides on otherwise bio-inert SAMs as a useful mechanism to study the influence of ECM-derived peptides on cell behavior in a chemically well-defined background.

2.2.4 Spatially patterning ECM-derived biomolecule immobilization on SAMs—

Numerous methodologies have been developed to spatially pattern peptide immobilization onto SAM-based cell culture substrates. For example, patterned photochemical deprotection of SAMs terminated by nitro-veratryloxycarbonyl (NVOC)-protected hydroquinone provides spatial control over hydroquinone presentation and, in turn, location of peptide immobilization *via* the Diels–Alder mechanism described above.⁶⁵ Latent hydroquinone moieties can be electrochemically converted into reactive quinones to allow for spatial control over RGD immobilization and, in turn, cell adhesion onto a SAM.⁶⁶ Microfluidic approaches have also seen widespread applicability in patterning peptide immobilization on SAMs. For example, Mrksich and colleagues used a Y-channel microfluidic network that allows diffusion-mediated mixing of RGD and inactive RDG to form RGD gradients on a SAM-based cell culture substrates presenting maleimide groups.⁶⁷ Yousaf and colleagues demonstrated that passing pyridinium chlorochromate through microfluidic channels applied onto alcohol-terminated SAMs provided site-specific reduction of alcohols to aldehydes. In turn, these aldehydes reacted with amine-terminated RGD to form patterned cell adhesive domains on the SAM.⁶⁸ We recently developed a localized SAM replacement approach

within microfluidic channels to pattern peptide immobilization⁶⁹ (Fig. 4). In this approach, 1) a poly (dimethyl siloxane) microfluidic device is placed onto a bio-inert SAM comprised of triethylene glycol-terminated alkanethiolates; 2) a solution of sodium borohydride is added into channels within the microfluidic device, where this solution contacts the underlying SAM and induces removal of alkanethiolates to reveal bare gold; and 3) an aqueous solution containing chemically reactive alkanethiolates is added into the microfluidic channel, resulting in formation of a new SAM presenting reactive chemical groups. Our results demonstrate that we can pattern multiple cell adhesive regions within a single SAM, and the resulting substrates can be used to screen for the effects of RGD ligand density on mesenchymal stem cell behavior.

2.2.5 Orthogonal chemistries to immobilize different peptides onto a single SAM

—Collectively, the examples described in the previous section demonstrate that covalent immobilization of a protein or peptide onto a SAM provides a useful strategy to characterize the influence of a single biomolecule on cell function. However, cells possess multiple, distinct receptor types, each of which demonstrate unique biomolecule binding affinity and/or specificity.⁷⁰ Thus, chemically well-defined cell culture substrates that present multiple, distinct biomolecules may more appropriately mimic natural ECMs. In addition, SAMs with multiple biomolecules may enable studies of the concerted role of different biomolecules on cell function. Recently, we developed a strategy to covalently immobilize two distinct peptides onto a single SAM *via* orthogonal chemical reactions.⁷¹ In particular, we prepared ternary mixed SAMs consisting of alkanethiols terminated by tri(ethylene glycol), carboxylated-hexa(ethylene glycol) and azido-hexa(ethylene glycol) groups (Fig. 5). An amine-terminated peptide selectively reacted with surface carboxylate groups, while an alkyne-terminated peptide selectively reacted with surface azide groups. By varying the mole fraction of carboxylate and azide alkanethiolates during SAM formation, we could control the surface density of each group on the resulting SAM. In turn, we could independently control the density of two different peptides on a single SAM chip. We then used these substrates to characterize the influence of an integrin-binding peptide (RGDSP) and a heparin-binding peptide (TYRSRKY) on human mesenchymal stem cell adhesion. Our results demonstrated that these peptides work synergistically to enhance hMSC spreading under serum-free culture conditions. In contrast, the presence of serum-borne heparin disrupted interactions between cell-surface proteoglycans and the SAM, resulting in decreased cell spreading.

2.2.6 Summary—In each of the examples discussed thus far, a primary motivation was to provide a cell culture platform that promotes one or more specific biomolecular interactions, yet resists non-specific protein adsorption. By addressing the complications associated with random, non-specific biomolecule adsorption onto traditional cell culture substrata SAM-based cell culture substrates have provided a series of important single-factor correlations, such as adhesion ligand density *versus* cell adhesion. In addition, early examples demonstrate that SAM-based cell culture substrates can probe more complex correlations, such as the concerted influence of multiple, distinct cell surface biomolecules on cell adhesion. The latter examples move closer to mimicking biological systems, in which several distinct cell surface receptors are activated simultaneously. However, biological

system also feature a variety of non-covalent “sequestering” interactions that play a critical role in defining cell function. Thus, the following sections will focus on SAM-based cell culture substrates that feature non-covalent interactions.

2.3 Non-covalent immobilization of biomolecules onto SAM-based cell culture substrate

Non-covalent interactions are prevalent in biological systems, and recently a variety of non-covalent interactions have been used to impart SAMs with specific bioactivity. For example, Barbosa and co-workers demonstrated that immobilization of an IgG Fc domain antibody onto a SAM allows for site-specific, non-covalent immobilization an Fc domain and jagged-1 fusion.⁷² Jagged-1 is a ligand for cell surface Notch receptors involved in cell-cell juxtacrine signaling,⁷³ and Jagged-1 immobilized onto a SAM *via* this mechanism activates Notch-dependent signaling cascades, as shown by up-regulation of Hes-1, a Notch family target gene. In a separate approach, chelation of metal ions by proteins bearing polyhistidine tags, a strategy initially developed to purify recombinant proteins from cell lysates,⁷⁴ has been used for site-directed, non-covalent immobilization of growth factors onto SAMs. In particular, nickel-terminated SAMs sequestered polyhistidine-terminated recombinant epidermal growth factor in an appropriate orientation to initiate EGF receptor-mediated intracellular signaling cascades and increased proliferation of neural stem cells.⁷⁵ The specific, non-covalent interaction between carbonic anhydrase (CA) and benzenesulfonamide, a CA inhibitor, has also been used to non-covalently immobilize an RGD peptide onto a SAM.⁷⁶ Here, RGD-terminated CA binds specifically to benzenesulfonamide-terminated SAMs and mediates cell adhesion onto the substrate. In addition to these non-natural non-covalent interactions, non-covalent interactions observed in nature have also been used to alter the bioactivity of SAMs. For example, albumin-coated substrates are resistant to platelet adhesion,⁷⁷ and albumin demonstrates selective non-covalent binding to long-chain fatty acids.⁷⁸ Ratner and co-workers immobilized alkyl chains containing 18 carbon atoms onto OEG SAMs, and demonstrated that albumin specifically bound onto these SAMs rendering them resistant to platelet adhesion.⁷⁹

The natural extracellular matrix (ECM) is assembled through specific, non-covalent interactions between a variety of biomolecules, including proteins, proteoglycans, glycoproteins, and glycosaminoglycans. In turn, ECM biomolecules localize and regulate the bioactivity of soluble factors, such as growth factors. These observations from nature have recently inspired the development of SAMs that can harness the bioactivity of endogenous biomolecules to influence cell behavior. For example, Thomson, Smith and co-workers relied on a SAM-based cell culture substrate to demonstrate that heparan sulfate proteoglycans (HSPGs) secreted by mouse embryonic feeder layers are an important regulatory co-factor for embryonic stem cell expansion.⁸⁰ In particular, they used SAMs presenting covalently immobilized fibroblast growth factor-2 (FGF-2), a heparin-binding protein, to demonstrate that HSPGs from feeder layer conditioned medium mediate FGF-2 binding to the surface of embryonic stem cells. Cells in unconditioned medium lacking HSPGs did not adhere to SAMs presenting FGF-2, while cells in conditioned medium adhered to the substrate as a result of the ternary non-covalent interaction between immobilized FGF-2, HSPGs in the medium, and FGF receptors on the cell surface.

We have recently used heparin-binding SAMs to demonstrate that heparin at the cell-material interface is a key mediator of endothelial cell response to a soluble, recombinant growth factor supplement. SAMs presenting a biomimetic heparin-binding peptide can sequester heparin from fetal bovine serum, a common cell culture supplement. In turn, sequestered heparin mediates specific sequestering of a heparin-binding growth factor, fibroblast growth factor (FGF)-2, onto the SAM. Human umbilical vein endothelial cell (HUVEC) proliferation on heparin-binding SAMs in medium supplemented with recombinant FGF-2 was significantly enhanced when compared to HUVEC proliferation on SAMs that do not bind heparin. The extent of enhanced HUVEC proliferation was dependent on both FGF-2 concentration and the surface density of the heparin-binding peptide. Interestingly, in view of the reversible nature of the interaction between FGF-2, serum-borne heparin and the heparin-binding peptide immobilized on the SAM, this observation suggests an affinity-driven mechanism to locally amplify growth factor activity. Clearly identifying a mechanism such as this would be difficult using traditional cell culture substrates due to the presence of random, non-specifically adsorbed biomolecules alongside substrate-bound heparin.

We have also recently used heparin-binding SAMs to study, and amplify, endogenous growth factor sequestering in stem cell culture. Our results described above with HUVECs, along with studies from many other groups, have relied on supraphysiologic growth factor concentrations to influence stem cell behavior. Although useful for manipulating cell behavior *in vitro*, supra-physiologic growth factor concentrations likely provide limited insight into the influence of locally amplified growth factor activity within natural microenvironments. We hypothesized that heparin sequestered at the cell-material interface could locally amplify endogenous growth factor activity, analogous to the role of heparin in the natural ECM. Our results demonstrated that hMSC proliferation was enhanced on heparin-binding SAMs in standard cell culture medium, and this enhanced hMSC proliferation was due to amplified FGF-signaling. The enhanced hMSC proliferation on heparin-binding SAMs was sensitive to the volume fraction of serum in the cell culture medium, which contains both heparin and FGFs. In addition, hMSCs cultured overnight on heparin-binding SAMs in medium supplemented with FBS, then cultured in serum-free medium supplemented with a supraphysiologic concentration of FGF-2 also demonstrated enhanced proliferation. This observation suggests that heparin sequestered during overnight culture in serum can enhance hMSC proliferation by locally amplifying FGF-2 activity in the absence of other serum-borne factors. In addition, the enhanced stem cell proliferation observed on heparin-binding SAMs was equivalent to enhanced proliferation induced by recombinant FGF-2 supplements 1 ng/mL. Interestingly, we also observed that hMSCs cultured on identical heparin-binding SAMs in osteogenic induction medium demonstrated a significant increase in alkaline phosphatase activity, and this increase was dependent on signaling by bone morphogenetic proteins, another heparin-binding growth factor family. Together, these results demonstrate that heparin sequestered at the cell-material interface can influence distinct stem cell behaviors by locally amplifying the activity of different endogenous growth factors. These results also suggest that sequestering specific subsets of endogenous biomolecules at the cell-material interface may provide an important alternative to recombinant or xenogeneic biomolecule supplements to influence stem cell behavior. In

addition, this approach may be advantageous for developing *in vitro* culture models that more closely mimic native microenvironments, as well as designing materials that can harness the bioactivity of endogenous biomolecules to influence cell behavior for tissue engineering applications. It is noteworthy that heparin and heparin-binding ligands have also recently been immobilized on hydrogel substrates,^{11c, 81,82} which suggests the possibility to extend SAM studies to three-dimensional contexts.

3. SAM Limitations and practicalities

Despite the unique advantages of SAMs on gold as cell-culture substrates, they are also often cited as non-ideal cell culture substrates due to their limited stability under standard culture conditions, the difficulty associated with alkanethiol synthesis, concerns related to the quality of the underlying gold layer, and challenges related to SAM formation and handling. However, our group recently demonstrated that patterned cell adhesive domains that were formed *via* a microfluidic patterning approach within a bio-inert tri(ethylene glycol) background were stable for more than 14 days under standard culture conditions.⁶⁹ In addition, the prospect of SAMs as cell culture substrates has led to the emergence of numerous commercial sources for gold substrates that address issues related to substrate quality control, as well as numerous sources for alkanethiols that alleviate the limitations associated with chemical synthesis. SAM formation is also a relative simple and robust process: gold substrates can be immersed in aqueous or ethanolic solutions of alkanethiols for a few hours to a few days, resulting in the formation of reproducible, well-packed monolayers. Finally, the benefits afforded by chemically well-defined cell culture substrates that can definitively isolate and characterize individual classes of cell-ECM interactions likely outweigh their limitations, especially in view of the observation that many critical cell-ECM interactions occur on the time-scale of hours, rather than days or weeks. However, emerging innovations will likely provide further improvements in the simplicity and adaptability of SAMs, and enable multi-disciplinary investigators to address complex hypotheses.

4. Conclusion

The natural ECM presents multiple insoluble cues and localizes the activity of multiple soluble factors that influence cell behavior. Traditionally, studying the role of the ECM on cell behavior has involved adsorbing one or more ECM-derived biomolecules onto polymeric or glass substrates, and subsequently seeding cells onto this adsorbed protein layer. However, the adsorption process onto glass or polymeric substrates is random and non-specific, and the density of adsorbed biomolecules often does not correlate with their concentration in solution. In addition, turnover of the biomolecules bound to the substrate, either by desorption or enzymatic degradation,⁸³ can alter the type and density of biomolecules presented to a cell during the course of an experiment. To address these limitations, SAMs of alkanethiolates on gold have recently emerged as a useful class of chemically well-defined substrates that can be adapted to address diverse hypotheses related to cell-ECM interactions. In particular, the facile altering of SAM surface properties by changing the molecular head-group has allowed for well-defined studies on the correlation between surface chemistry and protein adsorption. In turn, these surfaces have provided a

unique platform to probe the influence of protein conformation on cell adhesion, migration, proliferation, and differentiation. In addition, SAMs presenting a wide variety of reactive functional groups have enabled covalent immobilization of ECM-derived proteins and peptides to study their influence on cell behavior in a chemically well-defined background. More recently, the observation of non-covalent sequestering within natural microenvironments has inspired the development of SAM-based culture substrates that can non-covalently sequester specific subsets of endogenous biomolecules to characterize and exploit their influence on cell behavior. The prospect of studying and regulating endogenous signaling using well-defined SAMs may lead to new paradigms in biomimetic cell culture. Taken together, the diverse examples featured in this review establish SAMs on gold as a unique and indispensable tool to elucidate the mechanistic details governing cell-ECM interactions.

Acknowledgments

This work was supported by the National Institutes of Health (R01HL093282 and R01EY017367) and the National Science Foundation (DMR 0906123).

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Fig. 1.

SAMs form spontaneously in the absence of external stimuli. Exposing a gold-coated substrate to an aqueous or ethanolic alkanethiol solution (left) results in alkanethiol adsorption, ultimately resulting in the formation of a well-packed, ordered monolayer on the substrate (right).

Fig. 2.

SAMs can be used to study the influence of an adsorbed protein on cell behaviors, such as adhesion, proliferation and differentiation. A) Schematic representation of cell adhesion onto a functionalized alkanethiol SAM that is mediated by an adsorbed protein. B)

Fluorescent photomicrograph of human mesenchymal stem cells adhering to a SAM (blue = nuclei, orange = f-actin, green = vinculin).

Fig. 3. Biomolecules can be covalently immobilized onto SAMs presenting reactive functional groups. A) Schematic representation of an alkyne-terminated cell adhesion peptide conjugated to an azide-terminated SAM *via* copper(I)-catalyzed azide-alkyne cycloaddition. B) Plot demonstrating that the surface density of the azide group correlates to the surface density of immobilized peptide. C) SAMs presenting different surface densities of RGDSP can be used to probe the correlation between adhesion peptide surface density and hMSC adhesion. Reproduced from 64.

Fig. 4. Biomolecule immobilization on SAMs can be patterned. A) Schematic representation of a "localized SAM replacement" approach. A microfluidic mask (blue) was added to a preformed SAM (2), which allows for selective exposure of the SAM to a destabilizing solvent (3), followed by addition of new alkanethiolates for new SAM formation (4), then localized covalent immobilization of a cell adhesion peptide (red) (5). B) Fluorescent photomicrograph and fluorescence intensity profile of a SAM that was patterned to present different densities of RGDSP within different domains. C) hMSC adhesion is confined to patterned domains presenting the RGDSP cell adhesion ligand. Reproduced from 69.

Fig. 5.

Orthogonal chemistries allow for conjugation of peptides with distinct biochemical activities onto a single SAM. A) Schematic representation of SAMs presenting orthogonally-reactive carboxylate and azide groups, which enable chemoselective immobilization of amine- and alkyne-terminated peptides, respectively. The surface density of the carboxylate (B) or azide (C) group is correlated to the density of amine- or alkyne-terminated peptide immobilized onto the SAM. D) The influence of an integrin-binding peptide and a heparin-binding peptide on hMSC adhesion (measured as projected cell area) can be probed using orthogonally-reactive SAMs by allowing for the surface density of each peptide to be controllably varied. Reproduced from 71.