



Surface functionalization and dynamics of polymeric cell culture substrates

John D Krutty¹, Samantha K Schmitt², Padma Gopalan^{2,4} and William L Murphy^{1,2,3}

The promise of growing tissues to replace or improve the function of failing ones, a practice often referred to as regenerative medicine, has been driven in recent years by the development of stem cells and cell lines. Stem cells are typically cultured outside the body to increase cell number or differentiate the cells into mature cell types. In order to maximize the regenerative potential of these cells, there is a need to understand cell–material interactions that direct cell behavior and cell–material dynamics. Most synthetic surfaces used for growth and differentiation of cells in the lab are impractical and cost prohibitive in clinical labs. This review focuses on the modification of low cost polymer substrates that are already widely used for cell culture so that they may be used to control and understand cell–material interactions. In addition, we discuss the ability of cells to exert dynamic control over the microenvironment leading to a more complex, less controlled surface.

Addresses

¹ Department of Biomedical Engineering, University of Wisconsin-Madison, 53706, USA

² Department of Materials Science and Engineering, University of Wisconsin-Madison, 53706, USA

³ Department of Orthopedics and Rehabilitation, University of Wisconsin-Madison, 53706, USA

⁴ Department of Chemistry, University of Wisconsin-Madison, 53706, USA

Corresponding author: Murphy, William L (wlmurphy@wisc.edu)

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such as growth factors, and the extracellular matrix (ECM), which is tissue-specific and consists of insoluble proteins and glycosaminoglycans. Evidence in the last decade suggests that the properties of the insoluble microenvironment can guide cell behaviors such as adhesion, proliferation, morphology and differentiation [1,2,3,4]. The combination of these soluble and insoluble signals regulates cell behavior [5,6]. While insoluble matrix components can be derived from animal sources, clinical application of stem cells will benefit from chemically defined and animal-product-free culture materials to reduce potential immunogenicity and batch-to-batch variability [7]. In particular, stem cells can internalize components of animal-derived culture materials, which have been shown to cause anaphylactic reactions and the production of anti-serum antibodies in clinical treatments [8,9]. Understanding and selectively presenting parts of the *in vivo* extracellular matrix in a cell culture setting can potentially improve the success of stem cell therapies and help answer fundamental scientific questions.

The overwhelming majority of cell culture is performed on 2D polymer surfaces (*e.g.* tissue culture polystyrene), which can non-specifically adsorb serum-borne proteins and thereby provide adhesion sites for cells [10]. These plastic cell culture materials are low cost, scalable (*e.g.* T flasks, stacked cultures), and have low batch-to-batch variation. However, plastic cell culture materials typically cannot be used to control cell–material interactions. While a series of chemistries have been developed to coat plastic, glass, and metal substrates, achieving control over the cell–surface interactions and maintaining long-term substrate stability in cell culture conditions remains a challenge [11,12,13,14]. This concise review presents a subset of recent advances and challenges in the modification of polymer substrates to control cell behavior (Figure 1). We address surface functionalization of cell culture systems, emerging chemically defined surfaces amenable to biological functionalization, and the ongoing challenge of understanding and controlling time-dependent cell–surface interactions.

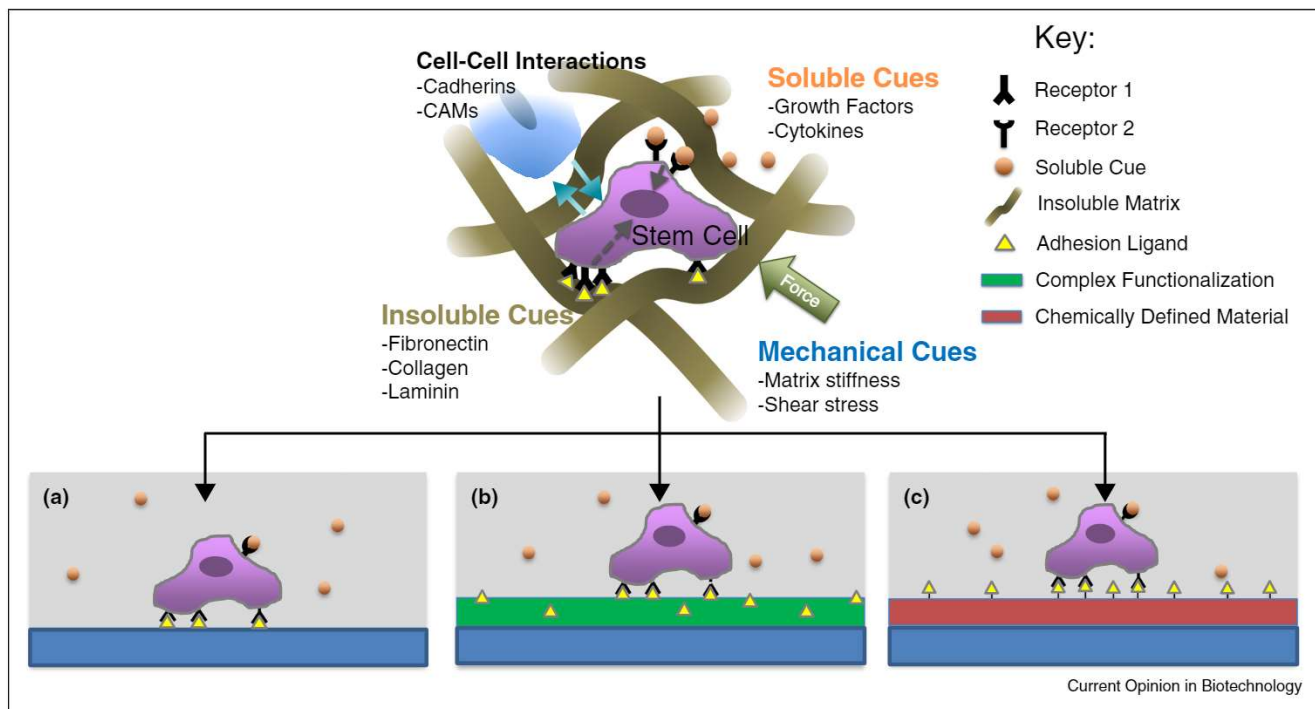
Introduction

The therapeutic potential of stem cells is leading to their increased use in clinical trials for treatment of wounds, disease, and as scientific models for drug discovery and toxicology. A major barrier to the reproducibility of cell-based treatments and models is the limited control over cell behavior. *In vitro*, cells interact with soluble signals,

Biological functionalization

Most mammalian cells are adherent, meaning they must be attached to a surface to survive. In order to achieve this adhesion, traditional cell culture relies on the adsorption of serum proteins from the media to the solid surface. Protein adsorption occurs within seconds through non-covalent interactions between the protein

Figure 1



Cell culture systems can reproduce aspects of the cell microenvironment. **(a)** Cells cultured on untreated TCPS adhere to adsorbed serum proteins and interact with soluble signals. **(b)** Complex biological functionalization adds insoluble cues derived from the extracellular matrix. **(c)** Chemically defined, functionalizable materials (red) enable tailored presentation of bioactive ligands.

and the material [10]. This use of adsorbed proteins to achieve cell-surface adhesion has the advantage of simplicity; however, it is not without drawbacks. Proteins that are adsorbed to a surface can denature, and may change in α -helix content, β -sheet content, and structural rigidity [15,16]. In addition, studies on the kinetics of protein adsorption in a two-component system are rare, dependent on many parameters (including diffusion, components, competitive adsorption, pH, temperature, and others) and are ineffective at accurately predicting adsorption in multi-component systems [17,18,19]. Thus, it is difficult to predict the concentration and conformation of adsorbed proteins in a cell culture environment.

Coatings that form the cell-material interface can also be composed of individually purified glycoproteins (*e.g.* fibronectin) or heterogeneous mixtures of proteins (*e.g.* Matrigel). The orientation of these adsorbed proteins is not easily controlled [20]. The availability of integrin-binding Arg-Gly-Asp (RGD) cell adhesion motifs in Fibronectin coatings, for example, is dependent on the surface chemistry of the material to which fibronectin has adsorbed. This, in turn, may influence cell adhesion and behavior [21,22,23]. A study by Ba *et al.* covalently anchored fibronectin *via* reactions through thiols or primary

amines on amine-functionalized polystyrene in an attempt to restrict the orientation of fibronectin on the surface. These reactions increased the retention of fibronectin to the surface, resulting in higher amounts of immobilized fibronectin and a resistance to deformation, but did not increase the availability of binding sites [24^{*}]. Klotzsch *et al.* used single-molecule imaging of fibronectin to track the relative distance between four available cysteines labeled with Cy3B and tracked using photobleaching and total internal reflectance spectroscopy. The average distance between the four sites increased from 33 nm to 43 nm upon fibronectin adsorption to glass in denaturing conditions, indicating a conformational change in the molecule. In the same study, the intra-label distance ranged from 24 nm to over 51 nm upon denaturing when fibronectin was adsorbed to glass that had been rendered more or less hydrophobic through plasma cleaning, pyrolysis, and chemical silanization [25^{*}]. Recently, Lin *et al.* demonstrated that the adsorption force between fibronectin and the underlying material can affect the morphology of cells grown on the surface [26^{*}]. Taken together, studies to date demonstrate that while protein coatings can enable cells to interact with a material, they do not allow for a high level of control over the orientation, density and availability of cell adhesion epitopes.

Synthetic biological functionalization of polymer substrates

To better control the concentration and identity of biologically active sequences on the cell culture surface, the polymer substrate can be modified with a non-fouling layer and short peptide sequences derived from ECM proteins — like collagen, fibronectin, and laminin — or growth factor mimicking peptides [2,3,27,28,29,30**]. A variety of synthetic approaches have been developed with a goal of generating cell culture substrates that: (a) require low concentrations of peptides to reduce cost and complexity; (b) are functionalized *via* peptide coupling chemistry which is efficient in aqueous media; (c) are scalable over large surface areas or complex geometries with uniform coverage; and (d) are stable during long-term (days-weeks) culture of cells. Modification of polymer substrates presents some additional, unique challenges when compared to modification of glass or metal substrates. Polymeric materials often must be modified in aqueous or ethanolic solutions, and in some cases cross-linked at low temperature or using UV irradiation in order to prevent damage to the polymer.

Many polymer substrates lack the reactive groups required for direct covalent functionalization with peptides. These substrates require additional functionalization steps, such as a plasma treatment, silanization or other chemical treatment, or coating with a reactive layer. Treatment of TCPS with allylamine, for example, adds primary amines to the substrate, which can then covalently couple to PEG-N-hydrocysuccinimide (NHS) containing copolymers to form a crosslinked coating [31]. Qian *et al.* used chemical vapor deposition to coat TCPS with an initiator molecule which initiated growth of poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH) brushes *via* atom transfer radical polymerization (ATRP) for embryonic stem cell culture [32]. This study compared the ATRP coatings to PMEDSAH polymerized by UV-ozone initiated free radical polymerization, first demonstrated in Nandivada *et al.* [33]. Lavanant *et al.* used a simple photobromination reaction to modify polyethylene and thereby polymerize polyethylene glycol methacrylate brushes in a water/ethanol mixture [34]. The resulting brushes were robust in a one month water stability test and facilitated cell adhesion through RGD peptides.

Coating a substrate with an insoluble layer may also provide an appealing method to introduce functional groups for subsequent non-fouling brush growth or peptide immobilization [35**,36]. An illustrative example of this is polydopamine which can coat many surface types and have been used for both zwitterionic polymer brush growth and peptide attachment [37,38]. In another coating approach, Schmitt *et al.* recently reported the synthesis of a PEG-based random copolymer in solution that was then spin-coated onto an untreated TCPS substrate

[28,29,30**]. The polymer coating contained azlactone functional groups for peptide attachment in aqueous solution at room temperature and low concentration (1 mM in aqueous solution) with no activation step. The resultant thin film permitted peptide surface density quantification (12.6 pmol/cm²) by X-ray photoelectron spectroscopy and RGD-mediated cell attachment.

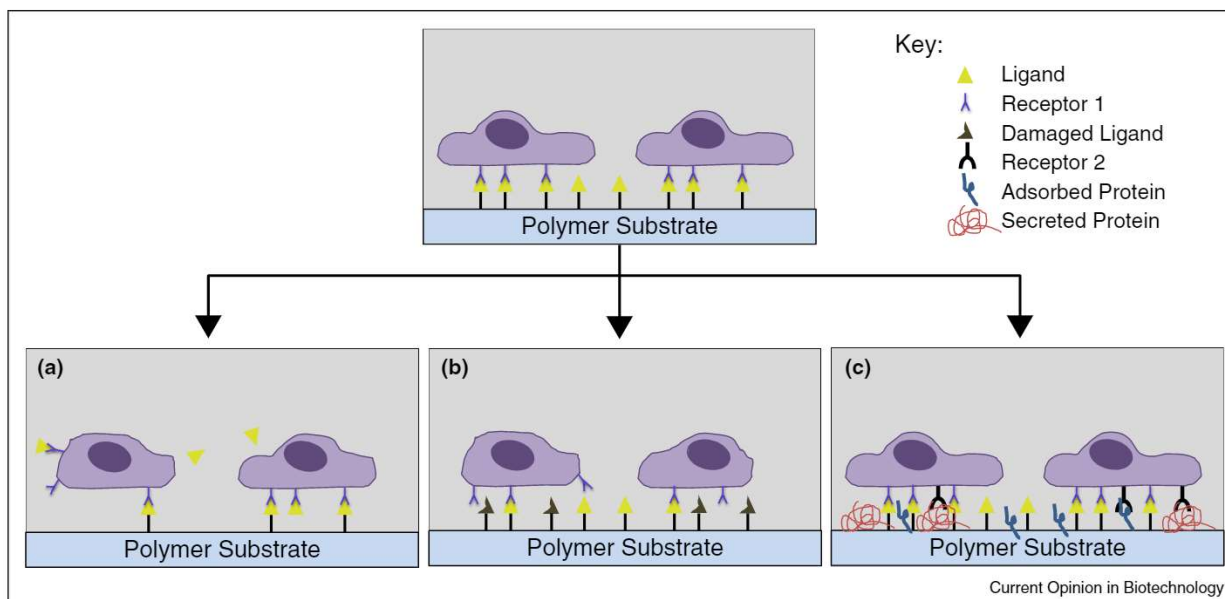
Time-dependent surface remodeling

In vivo, cells and the ECM are typically in a state of dynamic remodeling. *In vitro*, scientists have made significant progress in understanding and manipulating the initial characteristics of the cell culture substrate, prior to cell culture. However, despite the progress that has been made in generating and characterizing chemically defined surfaces for cell culture, there is still only a limited understanding of the dynamics of cell-surface interactions over time. In particular, while surfaces can be designed to present a single epitope (*e.g.* a cell adhesion peptide) for binding of cell surface receptors, it is not clear how long the cell has access to that epitope. Do cell adhesion epitopes rapidly become unavailable due to peptide-material bond scission, peptide instability, protein adsorption, cell-mediated ECM protein secretion, or other mechanisms (Figure 2)? This is an intriguing, poorly addressed question in cell culture applications. In the following paragraphs we introduce these dynamic mechanisms at the cell-material interface, and discuss the limited insights gained to date regarding the putative dynamics of cell-substrate interactions.

Common peptide-polymer bonds include amide, carbamate, alkyl sulfide, thioester, triazole, ester, thioether, and disulfide bonds, which may be vulnerable to hydrolysis, displacement, or protease-mediated degradation [39]. Carbamate [40], thioester [41], and ester [42] bonds are generally susceptible to hydrolytic degradation at physiological pH, while amide bonds are more stable [28,29]. Studies on the effect of polymer-peptide bond stability in cell culture systems are limited, although degradation of thioester, amide, and carbamate linkers has been observed on PEG thin films using cell-based assays [28,29].

In addition to cleavage of peptide epitopes from the culture surface, the peptide itself can be subject to degradation or damage in the culture environment *via* oxidation, deamidation, reduction and hydrolysis [43]. Methionine (Met), cysteine (Cys), histidine (His), tryptophan (Trp) and tyrosine (Tyr) are all subject to oxidation in the presence of oxygen radicals or alkaline pH [43,44]. Oxidation of these amino acids can also be triggered by visible light in the presence of oxygen, resulting in a decreased biological activity of the peptide, or, in the case of whole proteins, changes to the secondary and tertiary structure [45]. Met and Cys are susceptible to oxidation at their sulfur atoms, while His, Trp and Tyr

Figure 2



Over time, the cell–material interface can be dynamically altered through (a) removal of a cell-adhesive ligand from the surface, (b) degradation/denaturation of the ligand, and (c) fouling over time due to secreted or adsorbed molecules.

undergo oxidation of their aromatic rings [44]. In the case of cysteine, this can lead to unwanted disulfide bonds [46]. Glutamine and asparagine residues are susceptible to deamidation, resulting in isomerization or racemization of the residue [47,48]. Fragmentation of peptides *via* amide hydrolysis is possible, usually occurring at Asp-Gly and Asp-Pro sequences, but amide hydrolysis is not likely to occur near physiological pH (pH 7–8) [49]. Taken together, studies performed to date, and reviewed in more detail elsewhere [50], indicate that changes to the primary structure of peptides or whole proteins are likely to occur during cell culture on functionalized biomaterials. These changes can result in altered biological activity over time, which may result in unintended changes in the cell microenvironment. However, if changes in peptide and protein stability can be understood and harnessed they may provide adaptable mechanisms for intentional, transient presentation of cell–interactive epitopes.

Cell-mediated remodeling of synthetic surfaces is another source of dynamic variation. Many cell types have been shown to synthesize ECM molecules *in vitro*, including collagen, elastin, laminin, fibronectin, aggrecan, decorin, glycosaminoglycans, and calcium deposits. [51,52,53,54,55,56,57] These cell-secreted matrices can then be decellularized and used to direct stem cell differentiation [58,59,60], which provides evidence that cells interact with cell-secreted ECM molecules. Even in environments designed to be ‘non-fouling’ or ‘low-fouling’, protein adsorption is commonly observed [61,62], and degradation of the initially non-fouling

surfaces can reduce their ability to resist protein adsorption [63]. Cells can also dynamically modify their surrounding ECM by enzymatic degradation in concert with ECM molecule secretion. Cell-secreted or cell-associated matrix-metalloproteases (MMP) can mediate cell-mediated remodeling of the microenvironment *in vitro* [64,65,66,67,68]. However, the dynamic interplay of ECM protein secretion, protein adsorption, and cell-mediated ECM degradation is poorly understood. For example, quantitative understanding of how long a cell can engage with the initial substrate before cell-secreted ECM molecules or metalloprotease activity significantly alter the initial surface is lacking.

Concluding remarks

Recent studies have made significant progress toward controlling the initial cell–surface interface, and understanding the effect that this may have on cell behavior. While the initial conditions of these types of substrates are thoroughly examined, they may be susceptible to increasingly complex modifications after hours to days of cell culture. The quantitative characterization of cell secreted ECM molecules, protein adsorption through surface degradation, and degradation of functional peptides on cell culture materials is likely to be a highly significant area of study that is as of yet largely unexplored. In order to maintain control and biological relevance *in vitro*, there is a need to create cell culture surfaces that are designed for real-time characterization of polymer stability, peptide stability, ECM molecule secretion, protein adsorption, and cell-mediated ECM

degradation/remodeling. Ultimately, a clearer understanding of cell-substrate dynamics may lead to innovative approaches to dynamically adapt to cell behavior in a controlled and predictable manner.

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