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Review

Specific control of cell–material interactions: Targeting cell receptors using ligand-functionalized polymer substrates

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ABSTRACT

Cells respond to their environment in complex and sometimes poorly understood ways. Protein, peptide and synthetic peptidomimetic ligands may all be used to stimulate cells via receptor signaling, using interactions that are often highly specific. Polymer substrates that present these ligands provide a promising way to control cell development, both for applications in biotechnology and for fundamental studies of cell biology. Here we review a large range of techniques that have been employed to create and characterize ligand-functionalized substrates, with a particular focus on techniques that allow specific and consistent stimulation.

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Abbreviations: 2D/3D, two/three dimensional; AA, amino acid; AFM, atomic force microscope/microscopy; ATRP, atom transfer radical polymerization; BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CAM, cell adhesion molecule; CDI, 1,1'-carbonyldiimidazole; CNTF, ciliary neurotrophic factor; CTA, chain transfer agent; CuAAC, copper-mediated azide-alkyne click (reaction); DPN, dip-pen nanolithography; EBL, electron beam lithography; ECM, extracellular matrix; EDC/NHS, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide with N-hydroxysuccinimide activation; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; ERK/MAPK, extracellular signal regulated kinase/mitogen-activated protein kinase; FGF, fibroblast growth factor; FRET, Förster resonance energy transfer; GAG, glycosaminoglycan; GF, growth factor; GNP, gold nanoparticle; His-6, hexahistidine; HPLC, high performance liquid chromatography; IPN, interpenetrating polymer network; IR, infrared; L-DOPA, L-3,4-dihydroxyphenylalanine; LbL, layer-by-layer (deposition); LIF, leukaemia inhibitory factor; MMP, matrix metalloproteinase; nCP, nanocontact printing; NGF, nerve growth factor; NMP, nitroxide-mediated polymerization; NMR, nuclear magnetic resonance spectroscopy; NTA, nitriloacetic acid; PDMS, polydimethylsiloxane; PEG, poly (ethylene glycol); PI3k, phosphoinositide 3-kinase; QCM, quartz crystal microbalance; RAFT, reversible addition-fragmentation chain transfer; SAM, self-assembled monolayer; SANPAH, N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenyl-amino) hexanoate; SAP, self-assembling peptide; SMCC, succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate; SPR, surface plasmon resonance; TBTA, tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine; TGF- β , transforming growth factor beta; ToF-SIMS, time-of-flight secondary ion mass spectrometry; μ CP, microcontact printing; UV, ultraviolet; VEGF, vascular endothelial growth factor; XPS, X-ray photoelectron spectroscopy.

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1. Introduction

Receptors in the cellular membrane are a vital way in which cells can sense and respond to their local environment. Binding between cell receptors and their ligands, which may include proteins, peptides and synthetic molecules, is tightly controlled by physical, electrostatic and hydrophobic/hydrophilic interactions between the two. These interactions make ligand–receptor binding highly specific and therefore a prime candidate for tightly controlled cell stimulation.

Ligand-functionalized culture substrates are a promising method for achieving this stimulation [1]. The

advantages of using functionalized polymer substrates, compared to soluble ligands, vary with circumstance. They may include: receptors requiring a surface-bound ligand for activation (e.g., many adhesion signals); increased receptor stimulation time and concordant increased length of signaling period; ability to control ligand release over a period of time; ability to use smaller amounts of (sometimes quite expensive) ligand; and the ability to achieve continual stimulation in vivo without repeated dosing at high concentrations. Functionalized substrates may also be useful for creating simplified models of the in vivo microenvironment for the study of fundamental biological interactions, reproducible drug toxicology/efficacy

One letter amino acid codes

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
N	asparagine
O	hydroxyproline
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
Y	tyrosine

screening and for the generation of cells for use in therapy.

In this review we examine the myriad of techniques that have been used to create polymer-based substrates to target specific receptors. Relevant literature from other fields, including non-polymeric substrates, antibody and other diagnostic chips, nanoparticles, and soluble ligands has also been included where appropriate. We attempt to define general principles that can be used to make informed decisions when designing these substrates.

2. Biofunctionality

In this section we examine the suitability of different ligands for presentation from cell substrates. We also examine several ways in which attachment of a ligand to a substrate can change that ligand's biofunctionality, often (but not always) for the worse. The discussion here will also serve as background and justification for discussions on presentation methods later in this article.

Here we define a ligand to be biofunctional if it docks with a receptor, affecting downstream signaling. Note that under this definition, ligands may act as agonists (full or partial receptor activation) or antagonists (preventing agonists from binding, thus disabling the receptor). Many ligands can bind to multiple receptors, with each receptor having a different cellular function. Binding of short RGD-based peptides (arginine–glycine–aspartic acid; standard one letter amino acid codes will be used throughout this review) to integrin receptors is a common example; while different integrin receptors are known to have distinct cellular functions, the RGD motif binds to a large number of different receptors [2,3]. At the same time, it is important to note that receptors can also complex with multiple ligands; glycoprotein 130, a cytokine receptor, can bind to multiple cytokines, including ciliary neurotrophic factor (CNTF), interleukin-6, and leukemia inhibitory factor (LIF),

with each of these interactions producing different cellular outcomes [4].

Direct recommendations of any particular ligand or ligand type over another will not be given here, as in many cases it is up to the researcher to confirm that their ligand of interest is appropriate under their specific conditions. There will also be no tables or lists of ligands that have been previously immobilized; many lists or reviews of this type already exist, e.g., growth factors [5,6], RGD ligands [7,8], and short peptides [9]. While there are many examples using integrin-binding ligands (as a heavily investigated area of research) we aim to show general principles applicable to any signaling cascade.

2.1. Not all ligands are equal

2.1.1. Proteins versus peptides

When examining cellular interactions with ligand-functionalized substrates, there can be significant differences between the effects of a full protein and those of a smaller peptide that bears the same motif. This has been particularly investigated in the context of integrin-binding ligands such as RGD; however short peptides also exist that bind to other receptors, such as non-integrin-binding adhesion peptides and growth factor mimics [10–18]. Peptide sequences for non-receptor-binding functions such as growth factor binding peptides or enzymatic degradation sequences will not be discussed here.

For the purposes of this discussion, “peptides” will refer to those amino acid-based materials that are produced (using well-known synthesis protocols) by reacting amino acids (AAs) sequentially. In contrast, proteins are usually longer sequences that are either extracted or produced recombinantly. We recognize that alternative definitions are possible, and that some sequences of intermediate length may be produced by either method; in these cases, the functional properties of the resulting polypeptide may not fit cleanly into either category. Excellent recent opinion articles have also examined differences between proteins and peptides [19,20] and Table 1 further examines these differences in detail. Many of the functional differences between proteins and peptides can be described in a single comparison: complexity versus simplicity. However, deciding which of these is appropriate for a given study is a more difficult matter.

Peptides have many practical advantages over proteins: they are more resistant to environmental stresses, such as heat or solvents; they are easier to obtain in a single, chemically pure form; they are usually synthesized to contain only one or a small number of motifs, each with a defined function; and they are more easily modified to a different sequence or to include non-natural amino acids [20]. Peptides may be useful for elucidating the function of various parts of proteins, deconstructing their actions into tractable pieces (see also Section 4.3).

However no peptide system has yet come close to mimicking the well-adapted functional complexity, specificity and affinity of most proteins. Proteins often bind to their receptors with higher affinity than peptides due to well-defined tertiary structure. Proteins such as those found in the extracellular matrix (ECM) can contain large

Table 1

Comparison of protein versus peptide ligands.

Proteins (extracted or produced recombinantly)	Peptides (synthesised)
Functionality Binding sites show high specificity. Contain motifs for multiple receptors as well as growth factor-binding sites and other sequences. Probably contain as-yet unrecognised motifs. Motifs may be exposed by mechanical forces or proteolytic degradation. High affinity when correctly folded. Higher order structure from protein folding and dimerisation.	Small sequences may bind to multiple receptors, especially when very short, e.g. the tripeptide RGD motif binds to many integrins (although receptor-specific peptides do exist). Single function per motif, more easily analyzed results. Multiple motifs can be combined using fusion peptides or similar. No dynamic interactions within current peptide ligands. Generally lower affinity, although some cyclic peptides can bind strongly. Variable secondary and higher-order structures. Linear peptides may have more flexible conformation compared to cyclic ones.
Practicality Attachment points and orientation can vary in an uncontrolled manner when using non-specific coupling methods and chemistries. More difficult to modify for advanced attachment methods, particularly when using non-canonical AAs. May permanently denature in organic solvents.	Can readily be synthesised with a single functional group or motif for controlled, oriented attachment. Can be synthesised using amino acids that have protecting groups (for later modification) or non-canonical amino acids. Resistant to many solvents.
Production No molecular weight limit (large or multidomain proteins may be difficult to produce recombinantly). Lower cost per AA for large sequences. Susceptible to degradation. Often from animal sources. Different isoforms, glycoforms and spliceforms with variable activity. Contaminants when extracted.	Step-by-step manufacture limits molecular weight. Lower cost overall. More stable, particularly when cyclised. Synthesised. A single, chemically defined form. Chemically pure.

numbers of functional sites, and in many cases function in ways that are yet to be fully understood. These functional sites can provide not only receptor-binding sequences, but sequences which may be sensitive to protease degradation, which can unfold to reveal “cryptic” binding sites, which sequester growth factors from the surrounding environment, or which modulate structure or mechanical properties of the proteins [19,21], and these may affect receptor signaling. Many receptor-binding proteins also typically exist in glycosylated forms whose interactions are difficult to mimic using simple peptides.

Comparing specificity in proteins and peptides is difficult. Specificity in proteins can vary: while some growth factors commonly bind only to a single receptor, the multifunctional ECM proteins can participate in many simultaneous interactions. While this is advantageous when trying to elicit a strong cellular response, it can also make deconstructive analysis of downstream responses more difficult. In some circumstances, using protein domains may cut down the number of potential interactions, while retaining the higher affinity from the full protein [22,23]. On the other hand, although specific peptide ligands do exist for some receptors, short peptide ligands are often non-specific [19].

2.1.2. Improving peptide biofunctionality

Several types of modifications can alter the functional properties of small peptides, giving increased affinity and specificity. These modifications may stabilize the peptide's active site and its surrounding AAs into a conformation that promotes strong binding to the receptor and

subsequent activation. However, affinity may be decreased if the peptide is constrained into an unfavorable conformation [24].

Firstly, this can be achieved by adding additional amino acids on either side of the minimal binding motif (see also Fig. 1). As the combination of residues flanking these short motifs is modified, the peptide segment can adopt a more stable tertiary structure, which can increase cell adhesion [25] and specificity for different receptors [7,26] to RGD peptides. Such effects have also been observed for non-RGD ligands: Khew et al. showed that the peptide GFOGER (where O is hydroxyproline) needed to be presented in a helical formation (similar to collagen, from which the sequence is derived) in order to most effectively bind its target, $\alpha_2\beta_1$ integrin receptors. This structure was achieved by using longer peptide sequences surrounding the main motif [27].

Secondly, cyclized RGD peptides may also show increased specificity and affinity compared to analogous linear peptides [28], and this can enhance cell responses to biomaterials [29]. Cyclization can also prevent enzymatic degradation [24]. The degree of specificity and affinity of the ligand toward its receptor can be influenced by the number of amino acids, their sequence and use of D-amino acids [24,30]. Furthermore, the exact conformation of the cyclic ligand can alter affinity: for example, Assa-Munt et al. examined an RGD peptide that contained 4 cysteine residues, and showed that altering the pairing of the two disulfide bonds within the peptide could cause a tenfold change in its affinity for $\alpha_v\beta_3$ integrin receptors [31].

Fig. 1. (a) Minimal binding peptide motifs (e.g. RGD or IKVAV) have decreased affinity and specificity for receptors due to lack of stable structure (binding site conformation shown in blue). (b) Addition of flanking amino acids from the corresponding protein can increase structural stability and potentially induce electrostatic and hydrophobic interactions with the receptor. (c) Small cyclic peptides have very stable structures and can bind with affinities approaching that of whole proteins. (d) Protein domains e.g. the 10th Type III domain from fibronectin (image adapted from Protein Data Bank (reference code 1TTF)) have complex tertiary structure and strong binding to receptors, but are likely to be too large to be made via peptide synthesis. Full proteins (not shown) may have even more subtle interactions, for example involving glycosaminoglycans or the PHSRN synergy sequence in the 9th type III domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.1.3. Synthetic peptidomimetics

Receptors may also be activated by ligands that are not based on amino acid sequences. These types of molecules are rarely (and only recently) used for developing cell culture substrates but may be of use due to their potentially high specificity and affinity. Several studies have attached non-peptidic integrin-binding ligands to surfaces and assayed cell attachment [32–36]. Klim et al. modified a streptavidin-coated polystyrene surface with a synthetic compound which, in solution, was highly specific for $\alpha_v\beta_3$ integrin [33]. They found that attachment occurred almost solely through the $\alpha_v\beta_3$ integrin receptor, and downstream signaling pathways were activated, with the attached cells

forming focal adhesions. Rechenmacher et al. used agonists specific for $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins to selectively attach fibroblasts that expressed either of these integrins [36].

Although synthetic ligands are commonly used in other research areas, modeling may be required to ensure immobilization maintains the correct orientation and presentation of the binding domain when attached to polymer substrates. This area of research is little explored, but use of these ligands is likely to have several practical benefits, including stability and low-cost synthesis [35]. It is also likely that different reactive groups may be more easily incorporated into synthetic agonists than proteins.

2.2. Ligand–substrate interactions

2.2.1. Changes in conformation and binding site accessibility resulting from attachment to substrates

Attaching protein ligands to a substrate at multiple points (via adsorption or some covalent methods) can cause loss of biofunctionality through changes in orientation or conformation of the protein [5,37–41]. Importantly, the loss of bioactivity may not be complete, and can vary depending on the combination of attachment points in each individual protein. This makes quantitative studies of receptor activation virtually impossible.

This effect can also be seen in smaller peptides. Salinas and Anseth attached three similar RGD peptides to a polyethylene glycol (PEG) gel [42]. Dual-bound RGD encouraged slightly less cell survival than a singly-bound peptide of similar sequence, and significantly less than a singly-bound peptide with a spacer, possibly due to steric inhibition of binding. Binding a ligand to a surface using an amino acid side chain within the receptor-binding site, such as the lysine residue within an IKVAV peptide, can also prevent or reduce the strength of receptor binding [13].

Short chain molecules, typically based on PEG or polypeptide (often polyglycine) repeat units, are often used as spacers to improve ligand accessibility and prevent issues arising from steric hindrance from the underlying surface [7,8,43]. Degradable or stimuli-responsive spacers may also be used to allow release or activation of attached ligand at a later stage (see Section 4.4). There are no general rules on the length of spacer required, as this depends on the properties of the surface, ligand and receptor. Relevant concerns in choosing a spacer may include the location of the binding site on (or within) the receptor, surface roughness, distance of the ligand from the surface without a spacer, and any preference for flexible or rigid orientation of the ligand. A spacer that is too long may also decrease bioactivity; although the exact mechanism is not known, it is possibly due to shielding of the active site (as observed for ligands in solution) or changes in orientation caused by a flexible linker, or a lack of tension being induced during ligand–receptor binding (see Section 2.2.4 for further discussion of the relationship between tension and receptor binding) [7,44,45]. Pallarola et al. compared three different spacer types (alkane-, PEG- and polyproline-based linkers) of varying lengths, and showed increased cell binding to the inflexible polyproline spacers. Adhesion increased with spacer length for all types of spacer examined here (1–6 nm), despite shielding being observed from longer

spacers when binding was measured in solution, prior to attachment on the substrate [45].

2.2.2. Ligand–receptor complex internalization

Unlike adhesion ligands, growth factors are often internalized by the cell after they bind to their respective receptors. However, many studies have examined surfaces modified with covalently bound growth factors (that cannot be internalized) and have found that bioactivity may be retained, or altered [1]. In some cases, bioactivity (measured by overall cellular response) was even increased [46–49], possibly due to internalization of the growth factor being prevented [50].

However, care needs to be taken; several studies have shown that preventing internalization can alter or prevent signaling in some pathways, while leaving others unaffected. MacInnis and Campenot used microbeads with covalently coupled nerve growth factor (NGF) to physically prevent NGF internalization by sympathetic neurons [51]. A pro-survival pathway (PI3K/Akt) was activated, but another NGF signaling pathway that is associated with differentiation, ERK/MAPK, was not. Recent studies of interest have examined in detail how covalent linkages can affect the signaling of vascular endothelial growth factor (VEGF) [52,53]. Other studies have prevented internalization using various mutated proteins (that are involved in the internalization mechanism), and have also found cases where certain signals depended on internalization, while others did not [50,54].

2.2.3. Low fouling substrates

The use of low fouling substrates can prevent several problems that may arise when examining the effects of surface-bound signals. Proteins that non-specifically adsorb on the surface can modify the effect of the ligands of interest that were specifically attached. Receptors may be physically impeded from full access to the ligand under investigation, or alternatively other signaling cascades may modulate or override the intracellular signals of the ligand in question.

In addition, even when covalently tethered at a single position, proteins can still interact non-specifically with the substrate via ionic, hydrogen bonding or hydrophobic interactions, which may alter the protein's conformation [55,56]. By reducing the non-specific interactions between a tethered ligand and the substrate, protein resistant surfaces may increase the probability that the ligand of interest is available in a consistent, favorable conformation.

In general, the use of neutral, hydrophilic synthetic materials is the most common way to introduce low fouling properties into a substrate. These materials commonly include PEGs [9], polyacrylamide [57,58], polyglycerol [59], poly(hydroxypropyl methacrylamide/methacrylate) [60,61], poly(*N*-vinyl pyrrolidone) [62], or polymers which contain zwitterionic groups such as phosphorylcholines and carboxy- or sulfobetaines [17,63–65]. Naturally-occurring materials including hyaluronic acid and methyl cellulose have also been used to create thin films with some resistance to protein fouling [66–70]. Low-fouling materials may be used in the bulk (as hydrogels) [9,64,71], or to create surface coatings on other materials.

Many different methods exist with which to create low fouling surface coatings. The most common coating strategies employ the use of self-assembled monolayers (SAMs) comprising alkanethiols or silanes, or the grafting of polymer brushes, while several other strategies are also possible (see Table 2). SAMs are common due to their ease-of-use, however they suffer from limited long-term stability and are limited to metal (typically gold) or silica substrates [72]. A recent study showed significantly improved stability of ethylene glycol-based SAMs, showing resistance to cell adhesion (via adsorbed proteins) for up to 35 days [73].

Alternatively, polymer brushes can be either “grafted to” or “grafted from” polymer substrates: pre-formed polymer chains are “grafted to” substrates with a single step, whereas “grafting from” a substrate implies the polymerization of a monomer from a surface-bound initiator or chain transfer agent. In recent years, “grafting from” substrates using surface-initiated living radical polymerization techniques (in particular atom transfer radical polymerization (ATRP), but also reversible addition-fragmentation chain transfer (RAFT), nitroxide-mediated polymerization (NMP) and iniferter-based methods) have become far more popular, due to the possibility of obtaining high grafting densities and thick layers while retaining low polydispersity, and applicability to a large range of synthetic monomers [74–76]. “Grafting from” methods require an initiator or chain transfer agent (CTA) at the surface, from which polymerization is initiated; these can be incorporated into alkanethiols for use in SAMs [77], coated onto polymer substrates [78], or incorporated into polymers to create macroinitiators [79–81] and macroCTAs [82], which may potentially have broader applicability as cell substrates.

The exact mechanisms behind the ability of various substrates to reduce protein adsorption are not fully understood and are an area of active research [83,84]. Polymers and SAM layers that contain molecules which are polar, electrically neutral and contain hydrogen bond acceptor groups are generally required [85], although materials that have charge distributions within a small scale such as zwitterionic groups can effectively reduce non-specific protein adsorption. Hydrophilic materials are commonly used, as some models of protein adsorption cite the ability of certain materials to couple water “tightly” as the underlying mechanism of adsorption resistance. Other important properties include the density and packing of polymer chains, polymer brush structure, local osmotic effects and chain mobility [59,77,86]. It is possible that different mechanisms may be responsible for protein resistance associated with different polymers and polymer architectures.

It is important to note that “low-fouling” surfaces can vary in their ability to reduce protein adsorption; for example, star-PEG coatings allowed attached proteins to better retain their bioactivity compared to other coatings such as linear PEG or adsorbed bovine serum albumin (BSA) [55,56]. PEG-based polymers are also known to undergo oxidation (particularly in vivo or in biological fluids) which can impair their low-fouling performance [64,87,88], while betaine-based zwitterionic polymers may

Table 2

Surface coating techniques for creating low-fouling substrates that can be activated for ligand immobilization.

Coating method	Notes	References
Self-assembled monolayers (SAMs)	Commonly used to test attachment chemistries and to create simple 2D models for polymer substrates. Short chain PEG-thiols or PEG-silanes self-assemble onto a gold or silica substrate (respectively). A wide variety of functional molecules can be assembled into the monolayer for functionalisation. Simple formation. Easy to change functional groups for later ligand attachment. Cannot be used to functionalise polymers. Limited to 2D substrates. Limited robustness and long-term stability.	[95–98]
Polymer brushes (“grafting to”)	Polymer chains attached as preformed chains to a substrate. Excellent control over chain length and brush thickness. Limited brush density, which can increase non-specific interactions.	[56,70,99]
Polymer brushes (“grafting from”)	Polymer chains are polymerised from surfaces with appropriate functional groups. Several preparation methods available, including ATRP, RAFT, NMP and iniferter methods. Higher densities and better resistance to fouling than coatings via the “grafting to” method. Uses synthetic monomers, and multiple monomers can be co-grafted to create a functional brush surface. Low fouling brushes can stay repellent for several weeks under some conditions. Thick films still possible at higher brush density.	[74,75,95,100]
Interpenetrating polymer networks (IPNs)	Multiple monomers are polymerised into a single, intertwined network, each monomer providing different functionality (e.g. modulus variation, attachment of ligands, protein repellency). Independent control of modulus and ligand display.	[26,101,102]
Layer-by-layer (LbL) deposition	Multiple coating layers are built up via electrostatic, hydrogen bonding or covalent interactions. Both synthetic and natural polymers can be used in various systems.	[67,68,103]
Plasma polymers	Uses plasma to generate a highly crosslinked thin film from a volatile monomer. These monomers can contain functional groups useful in coupling reactions. Generally used with synthetic polymers. Good control over thickness. Applicable to a wide range of surfaces. Mechanisms and products not fully understood and varied reactive groups can be present in the final layer.	[85,104,105]
Lipid bilayers	Phospholipids self-assemble into a layer via hydrophilic/hydrophobic interactions. Functionalisation via the inclusion of functional components in the layer.	[106,107]

be more resistant to fouling, particularly in the long-term [64,89]. Several reviews are available that examine the use of both PEG and zwitterionic polymers as functionalizable, low-fouling substrates [17,90,91].

It should be noted that no surface has yet been shown to completely resist protein adsorption, and proteins can still interact with low-fouling brushes (and presumably hydrogels and other substrates) through various mechanisms, including diffusion through brush layers and adsorption to the underlying surface (if the brush layer is not sufficiently dense), adsorption at the brush–solution interface and entanglement within the brush layer [92]. Hence it is important that substrates are thoroughly rinsed after ligand conjugation. It is also noteworthy that cell survival and development often requires more than growth factors (in solution or on a scaffold) [93] or cell adhesion molecules (CAMs) [94] alone. When investigating these factors and using low protein fouling substrates, it may be necessary to co-immobilize an integrin binding ligand or similar, as ECM molecules expressed by cells will not adsorb in large quantities onto low fouling materials.

2.2.4. Mechanical properties of the substrate

The mechanical properties of the underlying material have also been shown to be able to modulate cellular behavior including cell attachment and spreading [108,109] migration [110,111] and differentiation [102,108,109,112,113] in a broad range of cell types. The mechanisms by which cells sense (through receptor

signaling) the mechanical properties of an underlying substrate/scaffold are biologically complex and an area of active research, and as such are partially outside the scope of this review; we refer the interested reader to the following reviews [114–117]. However, brief discussion of several key concepts related to material design is required.

Integrin receptors act as cellular mechanosensors; following the binding of the integrin receptor to an appropriate ligand, the actin cytoskeleton exerts tension on the receptor–ligand complex, and through these actin fibers, on the external environment. The tension through this connection causes proteins within the receptor to unfold and reveal hidden binding sites for proteins such as vinculin [114]. Vinculin in particular promotes the clustering of receptors (that are bound to ligands) into focal adhesion complexes, a vital intracellular structure in the cell adhesion process [114]. As such, if the external environment is not rigid enough, insufficient tension will be formed within the receptor for this process to occur; hence, integrin ligands on some (although not all) soft substrates have been shown to inhibit focal adhesion formation [109].

Several recent studies have thrown further light onto the mechanisms by which cells sense mechanical changes in the environment, and how controlling the mechanical properties of the substrate can influence receptor activation. Huebsch et al. examined ligand clustering related to variably stiff gels [118], while Trappmann et al. showed that the effects of changed modulus could also be replicated by attaching collagen at different crosslink densities

at a constant modulus [109]. Keung et al. examined intracellular signaling, in particular the role of Rho GTPases, in response to substrates of varying modulus [119] while Grashoff et al. examined tension within vinculin structures in focal adhesions [120]. These studies, along with many others, allude to the need to treat the integrin–ligand complex as a dynamic object that can sense and respond to many stimuli, not simply the presence or absence of an appropriate ligand. Further studies are needed to examine how nanoscale mechanical interactions influence cell behavior, and how control of these interactions can be built into cell-instructive scaffolds.

2.3. Biofunctionality conclusions

Ligand–receptor interactions are not simple on-off switches; they are complex signaling systems that can respond to a range of stimuli. Choosing an appropriate ligand with high specificity and affinity for a given receptor allows greater control over downstream signals and more straightforward or more detailed analysis of resulting cell behavior. Attaching ligands to a surface can modulate responses in several ways. Changes in the conformation or accessibility of the binding site will affect binding. Further activation of the receptor's signaling pathways can then also be affected by factors that are specific to each receptor, including mechanical properties of the substrate, requirements that the ligand be internalized for normal signaling, or interactions of signaling pathways with other proteins that are co-presented.

Surface-attached ligands are only one part of the entire cell environment, and both their ability to activate receptors and the effects of receptor activation are modulated by other components of that environment [121–123].

3. Presentation of ligands

A large range of techniques exists for presenting or modifying substrates with protein- or peptide-based ligands (see Table 3). These substrates can include 2D polymer surfaces, hydrogels, or 3D-architected materials like electrospun fibers, foams or sponges. Both synthetic and naturally occurring materials may be modified using an appropriate scheme. Several reviews have examined chemical strategies for immobilizing proteins and peptides onto

a surface, however these either focus on other areas of study such as nanoparticles [124] or protein biochips [125], or focus on different ligands that may be immobilized [1]. Activating receptors using ligand-modified substrates has additional complications that may not be as important in these other areas, but which are critical in cell–material interactions. These are discussed in Sections 2 and 4.

3.1. Protein and peptide hydrogels

Using protein- and peptide-based hydrogels as 3D cellular scaffolds provides a conceptually simple way to present receptor-binding motifs without any chemical modification. Matrigel is a complex mix of proteins purified from mouse sarcoma tissue, which has been successfully used as a cell culture substrate. It is composed of a mixture of laminin, collagen and entactin, along with a large number of other components. While this complexity may enable it to support cells effectively, its composition is poorly known and may vary from batch to batch, making deeper analysis difficult [126]. Other protein mixtures are also available that have similar complexity [127]. Gels made from purified ECM proteins such as collagen, fibronectin, laminin and vitronectin are also popular, and combinations of these proteins have been used to investigate the effects of different compositions on cell behavior [128,129].

Several different types of self-assembling peptide (SAP) are in common use as cellular scaffolds, and this area of study has been reviewed [130,131]. SAPs can also incorporate different signals, including RGD [132–134], other adhesion motifs [14,135] and growth factor mimicking sequences [16,136,137]. Sequences that bind heparin or bind growth factors directly have also been included into SAPs, and this is discussed briefly in Section 3.2.2. Peptide signaling motifs can also be incorporated into modular protein gel materials [138,139]. These materials are recombinantly produced proteins designed to contain multiple short peptide sequences that can control mechanical properties, degradation and growth factor sequestration as well as presenting receptor-binding sequences.

Both modular protein and SAP gels can also be designed to gel in vivo after implantation in liquid form, allowing the gel to mold itself to fit within a lesion prior to gelation, which may be of use in future applications. Gelation occurs via reversible shear thinning or in response to

Table 3

An overview of methods that are discussed within this review for presenting receptor ligands (typically amino acid-based, although not in all cases) from various substrates.

Expression within a poly/oligopeptide substrate	Adsorption	Affinity interactions	Covalent attachment
<ul style="list-style-type: none"> ECM protein gels Self-assembling peptide gels Modular protein gels 	<ul style="list-style-type: none"> Physisorption of e.g. proteins Advanced methods such as chemisorption of DOPA 	<ul style="list-style-type: none"> Growth factor affinity binding to ECM proteins Biotin/avidin binding His6/Ni²⁺-NTA Protein pairs Protein-binding peptide sequences (extracted from growth factors) Peptide sequences (discovered via phage display) Lectin-mediated binding 	<ul style="list-style-type: none"> Unmodified ligands <ul style="list-style-type: none"> via amines (lysine or N-terminus) via carboxylic acids (glutamic/aspartic acids or C-terminus) via thiols (cysteine) via other groups Modified ligands <ul style="list-style-type: none"> Click reactions Other advanced reactions UV-active functional groups

environmental stimuli such as temperature or ionic strength [131,140]. Compared to ECM protein gels, they provide the researcher with control over the signals being presented to cells, as they are far better defined than complex protein mixes, and are more likely candidates for therapeutic uses. While protein mixes are likely to remain the standard for some time for cell culture, due to strong cell interactions and ease of use, engineered peptide and protein gels are likely to provide a way forward for well-controlled stimulation of cell behavior.

3.2. Conjugation of unmodified ligands

3.2.1. Non-specific adsorption

Pretreatment of surfaces by passive adsorption has been used in many studies to activate surfaces with ECM proteins. This is a fast, simple technique that requires no knowledge of chemistry, and can be adapted to produce microarrays of a large number of spots, which can be used for combinatorial studies of ECM proteins and their interactions with cells [141]. It is without doubt the easiest technique available to modify substrates.

However using adsorption as a modification technique has several disadvantages. Most importantly, proteins attached via adsorption will change conformation due to their interaction with the surface, affecting biofunctionality. The orientation of adsorbed proteins and their active domains also cannot be controlled, and furthermore, any proteins present in cell culture media or in the *in vivo* environment may also adsorb onto a permissive surface, while at the same time, proteins of interest may desorb. This leads to a situation where the types of ligands, their density and the arrangement and biofunctionality of their binding sites are all unknown, giving a very poorly defined culture environment. For example, Garcia and coworkers have investigated the adsorption of fibronectin and fibronectin domains on surfaces presenting different functional groups, and showed that adsorption and later cell response was modulated by the changing surface [39,40,142]. Kohen et al. also showed changes in the structure of Matrigel after adsorption on various surfaces, which were related to subsequent changes in cellular response [41].

More advanced adsorption methods that attempt to address some of these concerns do exist [70]. For example, Messersmith and coworkers use adsorbed L-DOPA (L-3,4-dihydroxyphenylalanine) materials to modify surfaces for subsequent attachment of biomolecules [143,144]. L-DOPA adsorbs strongly to virtually any surface and is stable over long periods of time [78,145], making this method broadly applicable and stemming concerns about desorption.

Furthermore, recent work has shown that an appropriate choice of surface chemistry, followed by adsorption of ECM proteins, can provide an excellent substrate for long-term stem cell culture [146,147], demonstrating that an advanced level of control may not be required in some applications.

3.2.2. Affinity interactions

Many growth factors (GFs) and other cell-stimulatory molecules contain short peptide sequences that will

reversibly bind to ECM proteins, proteoglycans or glycosaminoglycans (GAGs). These functional sequences include heparin-, collagen- and fibronectin-binding domains, and provide a further non-covalent method by which ligands may be attached. These binding sequences can also be incorporated into fusion peptides or recombinant proteins alongside other ligands; we review this area separately within Section 3.3.3. These areas have been reviewed [148,149] and so only a brief overview and other related work will be presented here. A different affinity-based strategy which binds ECM proteins via lectin-mediated interactions, is discussed briefly in Section 4.4.3.

Heparin, a negatively charged GAG, is a particularly popular substrate for GF-sequestration, and can be incorporated into biomaterials both covalently and non-covalently [150]. Covalent methods use various reactions: for example, unmodified heparin may be crosslinked using carbodiimide chemistry [151,152], or modified to contain various functional groups [152–154]. Non-covalent methods have been developed that first covalently attach peptide sequences such as KRTGQYKL [155,156], TYRSRK [157], FHRIKA [25,158] or others [158–160] that are derived from heparin-binding domains in fibroblast growth factor (FGF) or other proteins. Heparin, and in turn growth factors, can then be sequestered from the extracellular environment, or can be bound prior to cell seeding/implantation for sustained release. A visual summary of various methods for functionalizing substrates with basic FGF (bFGF, the most commonly used GF in these studies) is shown in Fig. 2, encompassing studies on a wide range of synthetic polymer and peptide-based substrates. Heparin-binding peptide sequences can also be introduced into self-assembling peptides [161] and used to deliver growth factors [162]. In an interesting approach, Anderson et al. first immobilized heparin molecules that had been modified with photoreactive azides [52]. They then used the growth factor binding capabilities of heparin to orient VEGF in a presumably highly bioactive orientation, followed by ultraviolet (UV) irradiation to covalently “lock” the signal in place.

Growth factor binding domains can also be found on many ECM proteins [2,148], including collagen (for FGF) [163] and fibronectin (for VEGF) [164]. Using these mechanisms for GF sequestration may allow more specificity, as heparin binding domains are found in many different GFs. Further research is needed to examine how these GF-matrix interactions affect bioactivity and whether they can be used for engineering cell substrates.

Other smaller molecules have also been used to replace heparin. Lin and Anseth used the KRTGQYKL peptide sequence (discussed above) to directly sequester FGF, without the use of heparin as an intermediary [165]. Other sequences have been shown to sequester transforming growth factor beta (TGF- β) [166], while sequences that bind a growth factor directly have been included into self-assembling peptides [167]. Other groups investigated sulfonated peptides that were capable of mimicking the growth factor binding properties of heparin [168,169], and Maynard and coworkers later investigated substrates that use poly(styrene sulfonate)-based copolymers as a similar mimic [170,171].

Fig. 2. Strategies for affinity-based attachment of bFGF, a common heparin-binding growth factor. Heparin can be conjugated to a substrate covalently or via non-covalent methods such as electrostatic attraction, (a). Substrates can also be modified via affinity interactions with short peptides, (b). Heparin binding growth factors such as bFGF can also interact directly with peptides such as the KRTGQYKL sequence, (c), sulfonated peptides, (d), or with sulfonated synthetic polymers (e). Structures for bFGF and heparin adapted from the Protein Data Bank (reference codes 1HPN and 4FGF).

Using these methods, which mimic the mechanisms by which many growth factors are presented in vivo, has many advantages. Presentation of growth factors via ECM affinity interactions prevents any loss of activity and specificity, and can even modulate or enhance certain interactions [148,172]. Furthermore, no chemical modifications or mutations of the proteins are required. Sequestration also allows growth factors to be passively released over time [148], which may be an advantage over covalent binding in cases where internalization of the growth factor can be an important part of signaling, or when a timed release of growth factor is desired. Some of these systems, for example those involving heparin, also have the potential to interact with other growth factors, such as those released by cells. This could be either advantageous or disadvantageous, but needs to be kept in mind when designing studies.

3.2.3. Covalent attachment of ligands via amines or carboxyls

Amines are by far the most common target for covalent attachment of proteins, being found in lysine residues (one of the most common amino acids) as well as at the N-terminus. The high abundance of available, surface-exposed amine functional groups means that almost any protein may be covalently bound to substrates in this way without further modification via a range of different chemistries (Fig. 3). However, this method can also lead to poor site specificity, poor control over orientation

and/or multiple point conjugation, which can reduce biofunctionality in an unquantifiable way [5,37,173]. Some linker protocols may also risk the ligand self-crosslinking. The traditional coupling agents for amine conjugation are well known [174] and will not be reviewed here. Those in most common use include EDC/NHS (*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide with *N*-hydroxysuccinimide activation), CDI (1,1'-carbonyldiimidazole) and SANPAH (*N*-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenyl-amino) hexanoate). Many of these reagents are water sensitive, which can drastically lower yields, although evaporating the ligand solution during conjugation can be used to improve yields [67].

Coupling via acidic residues or via the C-terminus suffers from many of the same drawbacks. It is more rarely used, but a small number of studies have immobilized ligands to amine-presenting substrates and shown increased bioactivity compared to unmodified scaffolds [11,175,176].

However, in some circumstances, attachment via amines may be effective. In the case where a ligand contains no lysine residues (e.g., murine epidermal growth factor [37,46]), these methods may be simple yet effective. *N*-terminal specific reactions are available, which may alleviate many of the problems with amine-targeting strategies. However, many of these require specific amino acid residues at the terminus and are yet to be used to conjugate ligands to surfaces to create defined culture environments. One generally applicable protocol uses the altered pK_a of the terminal amine compared with those in lysine side-chains, performing the conjugation in an appropriate pH buffer to ensure that only the terminal amine is protonated. However, targeting of the *N*-terminal amine using this method is not perfect and ϵ -amines are likely to be modified as well [177]. One study describes a method for linking RGD peptides with *N*-terminal cysteines (at the free amine) via a phenoxy amino squarate reactive group, even in the presence of other free cysteines [178], while several other potentially useful techniques for targeting the *N*-terminus have been reviewed [179,180].

3.2.4. Covalent attachment via thiols

Thiols are a reactive group found on cysteine residues. Cysteine is a rare amino acid, and so unlike amines and carboxyls, most proteins contain few thiols, lessening problems related to a lack of site specificity [124,180]. Reactions with thiols are also specific and efficient in many cases.

However, the use of thiol reactions has a separate set of complications. For example, disulfide bonds between cysteine residues can be formed, which are often a key element in defining protein structure [173,180]. Also, they are commonly found within receptor binding sites and other active sites, and so conjugation in or near these domains may reduce biofunctionality. Finally, due to the aforementioned rarity of cysteine residues in many proteins, thiols may not be present in some proteins of interest. While this precludes the attachment of unmodified proteins, it is useful in strategies that introduce thiols specifically for conjugation (see Section 3.3.1).

A summary of reactions that have been used to conjugate peptide-based ligands via the thiol functional groups on cysteines is presented in Fig. 4. The most commonly

Fig. 3. Amine-targeting reactions for ligand attachment (blue sphere represents the ligand being attached). From top to bottom: reaction with carboxylic acid (typically via carbodiimides such as EDC) to form an amide bond; with a hydroxyl (via various crosslinking reagents) to form a carbamate; with an aldehyde (2 step via reduction of a Schiff base); with an isocyanate to form a carbamate; with a halogen (via nucleophilic substitution); with an epoxide; with an anhydride; and reaction of the amine in N-terminal cysteines with phenoxy amino squarate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

used reactions are Michael-type addition reactions with alkene-containing reactive groups such as maleimides, acrylolys and vinyl sulfones [181]. Maleimides are the most commonly used for immobilizing ligands to surfaces, either directly or to surface amines via a succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) spacer [26,49,182,183]. Thiol-ene photocoupling (a separate reaction also involving thiol groups reacting at carbon-carbon double bonds) has also become popular for spatially controlled immobilization of ligands [71,184]. Thiols also form permanent (debatably covalent) bonds with some metals, such as gold, which can be used to conjugate ligands onto gold nanoparticles within polymer matrices (see Section 4.1.3 for detail on this particular technique and its application). Another potentially useful chemistry is the use of a 2-pyridyldithiol-based crosslinker to form a disulfide bond [13,103]. Haloacetyl groups can also be used to bind thiols; although a range of crosslinkers is available [174], this reaction is only rarely used in this application [185].

Under certain conditions, conjugation via thiols may be a very good method for attaching ligands, in particular

when a single thiol can be targeted that does not play any known biological role.

3.2.5. Covalent attachment via other groups

Other techniques do exist which allow covalent coupling to other reactive groups, on amino acids such as tyrosine, arginine, methionine and phenylalanine [173,180,188]. These could also be used for surface functionalization. A recent study used tyrosine-tyrosine photocoupling with visible light to attach an RGD peptide to a resilin-based protein substrate [189].

3.3. Advanced conjugation with modified ligands

3.3.1. Introducing reactive groups

In recent years, many studies have examined chemical modifications of ligands that allow more advanced conjugation techniques. Short peptides and synthetic ligands can generally be manufactured to include almost any desired reactive group without major difficulty; however, proteins are more difficult to modify. Studies have used bifunctional linkers to modify free amines on the

Fig. 4. Thiol-targeting reactions for ligand attachment (blue sphere represents the ligand being attached). From top to bottom: Michael-type addition to a maleimide; thiol-ene photoclick reaction; reaction with gold surface or nanoparticle; reaction with 2-pyridyldithiol crosslinker; reaction with haloacetyl. Note that the Michael-type addition reaction can also be performed using vinyl sulfone or acrylate reactive groups, and that other thiol-based reactions are also available [186,187]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

protein, for example using SANPAH to create photoreactive ligands [69,190]. However, these approaches can also suffer from the same lack of site-specificity and changes in conformation as those techniques outlined in Section 3.2.

More recent work has created proteins with reactive groups at single, well-defined locations (often the N- or C-termini) via a range of methods. In the simplest case, extra cysteine residues may be added to the termini of proteins [191–193] for conjugation using chemistries from Section 3.2.4. In the case where there is only a single available cysteine at a terminus, this allows for control over orientation and prevents possible changes to bioactivity. As previously mentioned in Section 3.2.3, reactions that specifically target N-terminal cysteines are also available.

In recent times, a new range of reactions has become popular for conjugation of ligands to surfaces. These involve the use of “bio-orthogonal” reactive groups; groups that do not undergo notable side reactions with amines, thiols, carboxyls and other groups that are commonly present in a biological milieu [194]. These include groups such as azides, aryl azides, acetylenes, aldehydes and dienes, as well as chemical motifs such as biotin, which can be introduced site-specifically into proteins via a variety of techniques. These techniques have been well reviewed and include enzymatic reactions, site-specific ligation reactions, and the inclusion of non-canonical amino acids during protein expression [173,194–196]. Examples of the incorporation of specific functional groups into proteins (for surface attachment) will be discussed throughout this section, but are summarized in Table 4.

3.3.2. Advanced covalent coupling

The range of “click” chemistry reactions is of particular interest for attaching ligands [212]. The term “click” incorporates a family of reactions that display particular properties, including high specificity, stable linkages with orientation control and high yield under mild conditions (for ligand conjugation, ideally in aqueous solutions), many of which make them highly useful in materials design [213,214]. These properties align well with those desired in a ligand conjugation reaction, and many of the reactive groups used have some degree of bio-orthogonality. The group of reactions belonging to the click family is loosely defined, but includes thiol-ene click reactions (see Section 3.2.4), copper-mediated and copper-free (strain promoted) azide-alkyne cycloadditions, and Diels-Alder reactions. Many “ligation” reactions also share several of these properties and are sometimes included within the “click” family, and oxime ligations in particular have been used for conjugation of proteins and peptides to surfaces. These reactions require the introduction of functional groups not naturally found in protein or peptide ligands, with the exception of some ligation reactions and thiol-based reactions (although these may benefit from an introduced thiol, as discussed above). While this requires extra effort to introduce the groups, it gives the researcher full control over the site of conjugation. A range of different bio-orthogonal chemistries is discussed below, and summarized within Fig. 5.

The copper-mediated azide-alkyne click (CuAAC) reaction is the most well known of these. Studies have used both azide [215] and alkyne [157,216] functionalities to attach peptide ligands to a surface with the corresponding

Table 4

Modification of proteins to site-specifically include extra functional groups for surface conjugation. Modification of peptides is not included as it is presumed these can be incorporated during synthesis. Use of heterobifunctional linkers that were not site specific (i.e. using NHS esters, maleimides or similar) is also not included. See review by de Graaf et al. for further examples of the incorporation of non-canonical amino acids [173].

Reactive group introduced	Comments	Reference
Aldehyde	Enzymatic modification using formylglycine-generating enzyme. Requires an acceptor sequence within the protein Chemical modification of N-terminal amine using pyridoxal-5-phosphate	[197] [198]
Aldehyde/ketone	Incorporation of a non-canonical, amino acid	[173]
Azide	Enzymatic modification using protein farnesyl transferase. Requires an acceptor sequence within the protein Incorporation of a non-canonical, amino acid	[199] [200]
Acetylene	Incorporation of non-canonical amino acids	[173]
Biotin	Enzymatic modification using biotin ligase. Requires an acceptor sequence within the protein	[201]
Aryl azide	Incorporation of non-canonical amino acid	[202–204]
Diene	Heterobifunctional linker via expressed protein ligation (C-terminal specific)	[205]
Protein or peptide binding motifs (including hexahistidine tags, ECM-binding domains, polymer-binding sequences, etc.)	Creation of a fusion protein containing the signaling protein along with binding motif	[38,206–211]

Fig. 5. Bio-orthogonal chemistries for covalent ligand immobilisation. Dotted lines surround examples where the same reaction is used, but with the location of reactive groups reversed. (A) Copper mediated azide–alkyne click. Copper (I) is typically generated from copper (II) using a reducing agent such as sodium ascorbate. (B) Diels–Alder reaction. (C) Oxime ligation. (D) Photocoupling using an aryl azide. The most likely reaction pathway of several available is shown, which involves ring expansion followed by reaction with an amine or other nucleophile. However, other reactions also occur which do not require a nucleophile to be present or do not involve ring expansion.

functional group. Proteins have also been attached to surfaces via click-chemistry. Duckworth et al. used the enzyme protein farnesyl transferase to add an azide group to a protein for conjugation to a surface via CuAAC [199]. The protein was engineered to bear a four amino acid enzyme recognition sequence at the C-terminus. Sun et al. also reported the surface conjugation of an azide-functional protein which incorporated an azide-carrying methionine at the terminus [200].

The reaction requires copper as a catalyst, which might interact with some proteins in an unwanted fashion during the reaction, and may be cytotoxic even at low concentrations. Previous studies have found that copper can be removed with high efficiency using complexation with a chelating agent such as EDTA (ethylenediaminetetraacetic acid) [217], although complete removal from a surface is difficult to verify. Despite the reaction nominally proceeding to high yields, most studies so far have used large excesses of ligand. The reaction can be slow, although this can be improved by the use of a copper ligand such as tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine (TBTA) [218]. Strain induced azide alkyne click reactions have not been used for attaching receptor ligands as yet, although cyclooctyne functional peptides were used as a crosslinker in a hydrogel which also contained cells [219], illustrating that there were no apparent cytotoxicity issues associated with the reaction.

Diels–Alder reactions involve the conjugation of a diene to a dienophile [220]. These reactions are generally slower than other click reactions, but do not require any catalyst. The reaction is almost bio-orthogonal, however if using maleimides as the dienophile, care is needed due to potential cross-reactivity with cysteine [180]. Otherwise, the reaction is able to be performed in the presence of many other reactive groups, so, for example, Diels–Alder and CuAAC reactions can be used to co-immobilize two different ligands [221]. de Araújo et al. used expressed protein ligation to functionalize a protein with a diene reactive group. This protein could then be conjugated to a surface using surface maleimides as the dienophiles [205]. Mrksich, Yousaf and coworkers have published several studies examining the effect of adhesion peptides immobilized via Diels–Alder reactions, where hydroquinone groups on the substrate were oxidized (electrochemically or via photoactivation) to benzoquinones, which could then be targeted using a diene-functionalized ligand [222–224].

Quinone-activated substrates were also used in later studies for the attachment of oxyamine functional ligands using oxime ligation [225–229]. This reaction between a ketone/aldehyde and an oxyamine results in a stable bond and is not sensitive to aqueous reaction conditions. Oxime ligation was also used by Pauloehrl et al. for patterning of an RGD peptide, where a photocleavable group revealed an aldehyde upon deprotection [230].

The reaction has also been performed with the location of functional groups reversed, by modifying ligands with either ketone or aldehyde groups and conjugating to an oxyamine functionalized substrate. While there is potential for cross-reactivity with amines, in general the oxime reaction is heavily favored [194]. Maynard and coworkers have used this chemistry to immobilize

a ketone-functionalized RGD peptide [171]. Bertozzi and coworkers describe a method to introduce aldehydes at either terminus of a protein, using enzymatic conversion of a cysteine into formylglycine within a 6-residue sequence [197,231]. Gilmore et al. describe an alternative method to convert an N-terminal amine into an aldehyde using pyridoxal-5-phosphate [198]. This was later used to immobilize model proteins to substrates [232,233].

UV-mediated immobilization has been widely used in patterning studies, as detailed in Section 4.1.2. Aryl azides have also been introduced directly and site-specifically into proteins by incorporating p-azido phenylalanine, a non-canonical amino acid, into the protein sequence [204]. Carrico et al. immobilized a cellular adhesion protein of this type [202], while Ogiwara et al. immobilized a modified epidermal growth factor [203]. The “bind and lock” method of Anderson et al. (also see Section 3.2.2) is also of interest due to its ability to orient heparin-binding ligands prior to UV immobilization, as claimed by the authors [52]. UV-mediated reactions between acrylates have also been used to immobilize multiple ligands within a three-dimensional hydrogel [234].

Other chemospecific reactions such as Staudinger, tetrazine and hydrazone and native chemical ligations have also been used for protein conjugation, although not for the generation of ligand-functionalized substrates applied to cell culture [124]. Several other bio-orthogonal reactions are also promising for these applications [194,235].

3.3.3. Affinity interactions with modified ligands

The interaction between biotin and members of the avidin family of proteins produces the strongest known non-covalent bond (dissociation constant 10^{-15} for avidin) [125]. This interaction has several advantages for immobilizing ligands: biotin is a small molecule and considered unlikely to interfere with bioactivity when attached to a ligand, the interaction occurs under mild conditions, and if a single biotin is added, consistent orientation of the ligand can be achieved [125]. Attachment via biotin–avidin interactions can also allow much higher yields and resulting bioactivity compared to a similar conjugation using EDC/NHS chemistry [201].

Biotin–avidin interactions have been used in many studies to immobilize ligands to a surface [22,201,236–238]. Biotinylating the ligand can be done pre-translation for recombinant proteins [22], post-translation using bifunctional linkers [174], or in a site-specific manner using biotin ligase [201]. This interaction is compatible with and orthogonal to many other conjugation reactions [239].

Substrates are typically activated with avidin or derivatives such as streptavidin and NeutrAvidin™ prior to conjugation. These vary in several relevant properties including production method, cost and non-specific binding. Recent work showed that both use of NeutrAvidin and extra blocking may be required to reduce non-specific adsorption of proteins [240]. Surfaces are activated with avidins by adsorption or amine-targeting covalent linkages, as these methods are simple and can still allow effective biotin–avidin binding. Advanced conjugation methods are not generally used, although the method used for

avidin activation has been shown to affect subsequent biotin binding [99]. Alternatively, the substrate may also be biotin-activated, taking advantage of the avidins' ability to bind up to four biotins (making the final linkage biotin–streptavidin–biotin) [56,238].

Similarly, pairs of proteins which exhibit high-affinity, non-covalent binding such as human serum albumin/albumin binding domain [210] and barnase/barstar [211] were used by Wylie et al. to prepare substrates with attached proteins. The second study from this group showed that it was possible to accurately pattern multiple factors using different binding pairs (biotin/streptavidin and barnase/barstar), as the interactions of the different binding pairs are highly specific and mutually orthogonal [211], while further studies from the same group examined binding pairs with lower affinities for slow release studies (see Section 4.4.1).

Hexahistidine (His6) motifs are commonly used in the purification of recombinant proteins, but can also be used to bind proteins onto cell culture substrates. Typically a surface activated with nickel-nitriloacetic acid (Ni-NTA) is used [37,241,242], although one study used zinc due to concerns with the potential cytotoxicity of nickel [206]. Dissociation constants down to 10^{-13} have been reported [124]. Binding is easily reversed using a divalent metal ion, chelating solution or a molecule that can bind competitively to the NTA complex [125]. This method has been reported to give control over orientation of the ligand being attached [124] and can increase bioactivity compared to covalent immobilization via amines [37]. Other affinity tags may also be used to immobilize proteins [125,243].

As discussed in Section 3.2.2, certain amino acid sequences that are present in growth factors will bind to substrates in vivo such as ECM glycoproteins and GAGs. These sequences can also be adapted and used as ligands, so that, for example, a collagen-binding sequence may be included in a non-collagen-binding ligand for the purposes of attachment [208,244,245]. These types of peptide sequences were also reviewed by Hudalla and Murphy [148]. These generally have much weaker binding constants than those for either biotin–streptavidin or His6-Ni-NTA methods (10^{-7} to 10^{-8}) [201] and so may undergo a slow release. Unlike sequestration of unmodified ligands, it is unknown whether these systems show any enhancement of bioactivity from oriented presentation. Proteins including epidermal growth factor (EGF) have also been captured via coiled-coil interactions, using proteins that have first been modified to contain a coil domain. This method will also allow for control of orientation, depending on where the coil sequence is placed [38,204].

Peptide sequences have also been discovered that bind to synthetic polymer substrates such as polystyrene [209,246,247], poly(glycolic acid) [248] and polypyrrole [249]. These are discovered by phage display techniques that scan large libraries of peptide sequences for binding. A polystyrene-binding sequence had sufficient affinity for the surfaces that RGD patterns could mediate cell attachment for up to 1 week [246]. At this point most studies have examined adhesion peptide sequences, with the exception

of Konagaya et al., who attached a polystyrene-binding sequence to EGF and examined cell response on the modified polystyrene [209].

3.4. Presentation methods summary and conclusions

A range of techniques that have been used to attach ligands to polymer substrates is presented in Table 5. Ultimately, the great advantage of using surface-bound ligands is the specificity of their interactions with proteins and cell surface receptors. The method chosen to present a ligand can have notable effects on the activity of the ligand, e.g., on the strength and specificity of the interaction, as well as surface density. Control over ligand presentation is thus the ultimate goal of any immobilization method. An ideal ligand-binding protocol should attach ligands with reliable yield, with all ligands in an identical conformation and orientation, limiting variation in biofunctionality. The effects of permanent versus impermanent attachment (see Section 2.2) should be taken into account. Ideally, the reaction would also have relatively fast kinetics, be high yielding and work consistently in aqueous buffers.

As such, many traditional methods of attaching (particularly protein) ligands, which can result in ligands being presented in ill-defined or random conformations, are far from ideal. Methods that use more specific chemistries can require more effort and in some cases are still not routine. However, attachment through a single, well-defined linkage, using chemistry with few competing side-reactions, should allow much better control over biofunctionality than some older methods in the literature, and orthogonal chemistries may also allow for the independent attachment of multiple factors.

The advantages of permanent or impermanent immobilization are dependent on many variables, and particularly in the case of growth factors, are still under investigation. For permanent immobilization, older non-specific techniques lack control over the final orientation and conformation of the ligands being immobilized. Techniques using click-type reactions, or quasi-permanent attachment using high affinity non-covalent interactions can overcome some of these issues. Affinity interactions such as growth factor sequestration or lectin-mediated binding, which mimic interactions that occur in vivo, are also of particular interest for the creation of dynamic substrates.

4. Spatial and temporal control of ligand presentation

Spatial patterning of receptor ligands is known to affect cell behavior [274,275]. Clustering of adhesion ligands is important in the formation of focal adhesions [118], and patterning of ligands into clusters (while keeping constant overall density) has been shown to have a greater influence over certain cellular behavior than changing overall density [267]. Gradients in the concentration of either growth factors or adhesion ligands can encourage cells to migrate, polarize or send processes toward areas of higher ligand density [276,277].

Table 5

Overview of ligand conjugation chemistries.

Ligand reactive group	Surface reactive group (linker/catalyst/reaction type)	Notes	References (see also in-text)
Amine	Carboxylic acid (via carbodiimides, e.g. EDC ± sulfo-NHS)	Very commonly used reaction. Reagents sensitive to hydrolysis, but reaction can still be performed in aqueous solution. Has been shown in several studies to be detrimental to bioactivity of ligands.	[37,67,104]
	Any (via Sulfo-SANPAH)	Can be performed by first activating either the surface or the protein. Other amine- and thiol-reactive photocrosslinkers also exist	[69,108,109,250,251]
	Hydroxyl (via CDI, p-nitrophenyl chloroformate disuccinimidyl carbonate, succinic anhydride)	Reagents are generally sensitive to hydrolysis and reactions are commonly performed in organic solvents.	[46,252–257]
	Anhydride	Rarely used	[258]
	Epoxide	Fast and high yielding reaction. May also react with carboxylic acids.	[259]
	Halogen	Can be performed in aqueous buffers.	[260]
	Aldehyde	Aldehydes generated via oxidation of a PEG substrate	[261]
	Isocyanate	Highly water sensitive reagents.	[262]
	Phenoxy amino squarate	Targets only N-terminal cysteines.	[178]
Carboxyl Thiol	Amine (via carbodiimides)	Similar to attachment via amines, but more rarely used. Low efficiency coupling with some loss of bioactivity.	[11,113,175]
	Maleimide/acrylate/vinyl sulfone, or amine if via SMCC linker (Michael-type addition)	High yielding and moderately fast.	[48,103,107,183,193]
	Double bond (thiol-ene)	UV photocatalysed reaction (or radicals), not to be confused with Michael additions to similar groups. Specific and high-yielding under aqueous conditions.	[71,219,263]
	Dithiopyridine crosslinker	Forms a disulfide linkage that can be reversed using reducing agents. Homobifunctional linker which may require care to avoid self-crosslinking. Crosslinker has poor aqueous solubility, may require solvent [125].	[13,103,183]
Naturally occurring amino acid sequence	Metal e.g. gold	Used to conjugate adhesion peptides to gold nanoparticles.	[109,264–268]
	Haloacetyl	Rarely used for ligand coupling	[183,185]
	Heparin, other ECM components, sulfonated substrates	Peptide sequences discovered in growth factors, which sequester the GF to the ECM. Sequences used may occur in the ligand naturally or can be introduced (from other GRs) using recombinant proteins or during peptide synthesis.	[148]
Glycan	Glycan (lectin mediated binding)	May allow cells to dynamically remodel their environment	[269]
Alkyne	Azide (CuAAC)	Bio-orthogonal reactive groups. Copper catalyst, which can potentially be disruptive to cells and proteins if not completely removed. Theoretically highly efficient reaction but can be slow without catalyst, and most protocols use large amounts of ligand.	[157,216]
Azide	Alkyne (CuAAC)	Not used previously for ligand conjugation, but has been used for gel formation in the presence of cells. Removes the need for a copper catalyst.	[199,215]
	Cyclooctyne (Copper-free click)		[219]
Aryl azide	Amine or reactive hydrogen (effectively none required)	UV-activated linking of pre-activated ligands. Superior to methods involving SANPAH crosslinker due to site-specific incorporation of the photoactive group.	[202–204]
Diene	Dienophile e.g. maleimide (Diels–Alder)	Bio-orthogonal reaction. Slower than other click reactions [212]. If using maleimide as the dienophile, thiols in the ligand may require protection. Water is an ideal solvent for this reaction.	[205,221–224]
Ketone/aldehyde Oxamine	Oxamine (Oxime ligation)	Stable bond, nearly bio-orthogonal reactive groups (aldehydes may react with amines under some conditions), mild conditions, no catalyst required.	[171,232,233,270]
	Ketone/aldehyde		[225–230]
Acrylate	Acrylate	Used in 3D photopatterning	[234,271–273]
Biotin (also proteins with high-affinity partners)	Appropriate binding partner (e.g. avidin derivative for biotin)	Very high coupling yield under simple conditions. Binding partner must first be immobilised to the substrate, which can be achieved in a number of ways.	[99,201,210,211,240]
Hexa-histidine ("His6")	Ni or Zn activated substrate	Reversible binding. Possibly results in oriented attachment.	[37,206,207,209,241,242]
Peptide sequence	Synthetic polymers	Sequences are discovered via phage display, which bind to various synthetic polymers. Similar affinities available to sequences found in growth factors.	[209,246–248]

Table 6

Ligand patterning techniques that are able to achieve sub-cellular pattern resolution.

Technique	Resolution/smallest feature size	Process notes: throughput, serial/parallel patterning	Arbitrary patterns?	3D patterns?	Equipment notes	Substrates	General notes and ideal applications
Micro/nano-contact printing	1 μm standard, <100 nm with advanced stamp materials	High throughput (parallel) after the initial master is made	Yes	No	Cleanroom required for master template, otherwise setup is accessible	Applicable to rough or curved 2D substrates.	Co-patterning of multiple ligands is difficult. Changes of pattern require a new master. Otherwise, a very common method, easily accessible, low cost
Photopatterning	Typically 1 μm ; 80 nm with multiphoton laser ablation of a thin film	Serial or parallel, depending on method	Yes	Yes	Some techniques may require specialised equipment. Other techniques have been developed recently using easily accessible equipment	Can be applied to non-flat 2D and 3D substrates. Specialised substrate chemistry is often, but not always, required. Optically clear gels required for 3D patterning	The only technique that has been applied to pattern in 3D at sub-cellular resolution. Multiple techniques give versatility.
Electron beam lithography	100 nm shown, theoretically much smaller	Low throughput serial patterning	Yes	No	Expensive equipment and usually cleanroom	Specialised substrate chemistry required. Only 2D substrates.	Potential for very high resolution arbitrary patterns
AFM techniques	30–40 nm for biomolecules, 10 nm achieved in other settings.	Low throughput (serial but can be parallelised using arrays of AFM tips)	Yes	No	Basic setup accessible but can be difficult to optimise	Requires a very flat 2D substrate e.g. SAM on silicon wafer	Arbitrary patterns at high resolutions. Can be very slow.
Colloidal lithography	100 nm shown	Parallel	No	No	Some specialised etching equipment required	Only 2D substrates	Can pattern large areas quickly. Used for studies on integrin clustering.
Micellar lithography (with GNPs)	8 nm	Parallel	No, but patterns are highly regular	No	Specialised equipment required both for forming the micelles and patterning	Only 2D substrates	Low variation in ligand spacing, particularly for a bottom-up method. Has been used extensively for studies on individual integrin receptor spacing
Island-forming block copolymers and blends	~20 nm shown	Parallel	No	No	Relatively accessible	Patterning is an integral part of the substrate chemistry	Can pattern large areas quickly. Used for studies on integrin clustering.

Here we discuss techniques used to create two- and three-dimensional patterns of receptor ligands, and present a summary in Table 6. We discuss briefly the need to present particular ligands in close proximity to each other for optimum activation of receptors. We also discuss techniques that have been used to vary ligand presentation over time. In this section we limit discussion to patterning on a sub-cellular (<5 μm) scale. While patterning of ligands into larger microscale patterns (e.g., arrays for combinatorial screening of cell-substrate interactions [93,94,141] or substrates for controlled colony sizes [278]) is undoubtedly useful technology, we will focus on patterning ligands at a scale where the patterns can have a direct effect on single-cell signaling. We also limit discussion on topographical patterns, which are reviewed elsewhere [279].

This section will focus where possible on studies that have immobilized receptor binding ligands, rather than other commonly investigated proteins such as BSA or IgG; although we acknowledge the relevance of these studies to a methods-based discussion, there is not scope within this review to examine methods that have not been used to create cell culture substrates.

4.1. Two-dimensional patterning

Several recent reviews exist that cover spatial patterning of 2D substrates with peptides or proteins, with applications in cell culture substrates [274,280–284]. Here we will summarize these and include discussion of other recent studies.

4.1.1. Stamping

Microcontact printing (μCP) is a well-established and versatile method for ligand patterning [285–287]. A master pattern is generally created in silicon wafer or a similar material, typically using photolithography or in some cases electron beam lithography. A soft elastomeric stamp, typically made from PDMS (polydimethylsiloxane), is then molded from the master, cured and removed. The stamp is “inked” with molecules that are to be placed on the surface, either proteins/peptides or functional molecules to which proteins/peptides can later be attached. The stamp is pressed onto the surface and the pattern transfers through either adsorption or reaction with surface functional groups.

This approach is used for patterning on a micron scale, although several techniques can be used that allow sub-100 nm patterns to be created (known as nanocontact printing, nCP). These use harder materials in the elastomeric stamp that prevent sagging [285,288,289]. Stamping using these methods can pattern large areas easily and inexpensively, and can deal with many non-flat, curved and otherwise non-uniform surfaces. It is difficult to co-pattern multiple factors accurately, as the stamp cannot be perfectly realigned. Access to facilities for creating master patterns can be an issue for more biologically-focused labs [275], although once the master is made it can be reused many times.

Micro/nano-contact printing has been used in several studies investigating the effects of patterned integrin

ligands on cell attachment, spreading, focal adhesion formation and migration on self assembled monolayers [290–295]. In a typical approach, patterns of functional alkanethiols are created, the substrate is backfilled with protein-repellant PEG-thiols, and then ligands are attached through either adsorption [291,294] or covalent bonding [292]. Alternatively, proteins such as fibrinogen (an ECM component) [288] or EphrinA5 (a neuronal guidance protein) [296] have been patterned directly from the stamp; however, it is noted that a small subset of proteins could lose bioactivity during direct (as opposed to indirect) patterning.

4.1.2. Photopatterning and electron beam lithography

Using light (particularly UV light) to pattern ligands is another common approach used for spatial patterning and several different techniques exist for the creation of complex patterns [297]. Patterns can be formed by either masking or maskless techniques. Photomasks are created using etchable spun-coated resist layers, or by creating a reusable mask, while maskless patterning techniques include interference lithography, single laser beams and multiphoton laser stimulation [289].

Indirect patterning, where ligands are attached to a pre-patterned reactive group, can be achieved by activation of a protected group such as an *o*-nitrobenzyl derivative. Previous work has patterned receptor ligands on deprotected surfaces displaying carboxyls [298,299], aldehydes [230], ketones [227], oxamines [270], thiols [211,252], and quinones (via protected, then reduced hydroquinones) [223]. Christman et al. used a photoacid generator to reveal a Boc-protected amine [232], while Alang Ahmad et al. degraded PEG-based films to create aldehydes [261]. Masking can also allow direct patterning of ligands that contain photoreactive groups such as aryl azides [202], while recent work has demonstrated that the CuAAC reaction may also be controlled with photostimulation, although not currently with the resolutions being discussed here [300]. A visual summary of covalent chemistries used in ligand patterning studies is included in Fig. 6.

Tightly focussed single-beam laser sources can also be used for surface patterning [252], but to date these approaches have not been able to achieve resolution at sub-cellular scale. However, two- or multi-photon techniques can be used to improve the situation, creating 2D patterns of multiple ECM proteins at sub-micron resolution [301]. One recent study used multiphoton laser ablation to ablate grafted PEG films from a surface [236]. This technique could be used to fabricate arbitrary patterns with a resolution down to 80 nm, well below the diffraction limit of the laser, however the laser equipment used in this technique is rather rare and expensive. Two-photon irradiation has also made 3D post hoc patterning of optically clear hydrogels a possibility (see Section 4.2).

Several studies have also developed novel methods that require little in the way of specialized equipment, thus making them accessible to bio-focused laboratories. These techniques use illumination from standard fluorescence microscopes, along with various responsive chemistries, including nitrobenzyl ether cleavage

Fig. 6. A summary of selected covalent coupling chemistries used for UV-mediated patterning of ligands. Techniques are grouped according to common features. Photomediated reactions used to pattern ligands directly to a surface under UV irradiation are presented within the blue loop, while the remaining techniques use the indirect approach of patterned deprotection of reactive groups, followed by reaction with an appropriately functionalised ligand. A range of reactive groups can be revealed following removal of nitrobenzyl ether (left) or other protective groups (right). Dotted black lines group indirect techniques that reveal the same reactive groups following deprotection. Within the red loop are reactions that have been used with two photon stimulation to create three dimensional patterns within hydrogels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

[298,299], and another technique which uses a photo-bleaching reaction to attach a biotin–fluorescein conjugate to the surface [302]. They retain the ability to pattern at resolutions of approximately 1 μm , and in many cases can also create patterns of multiple proteins.

Alternatively, electron beam lithography (EBL) has shown potential for creating patterned surfaces with features that are smaller than those possible when using photopatterning. The major restriction on photopatterning resolution is the wavelength of the light used. Based on this criterion EBL could achieve resolution down to below the size of single receptors, although as yet this has not been demonstrated. Use of EBL for biomolecule patterning was reviewed by Kolodziej and Maynard [303]. While it offers access to arbitrary patterns at high resolution, EBL is also low throughput and high cost, may require a conductive substrate and may be unavailable to many researchers [303].

Pesen and Haviland were able to co-pattern fibronectin and laminin on polymer-coated silicon wafers, creating dots, rings and lines with feature sizes from microns down to 150 nm [304]. Patterning of PEG-based substrates has also been used, to create laminin stripes that affected cell polarization [305], or co-patterns of multiple (up to 4) peptides/proteins [170,171,239]. In an unconventional approach, Rundqvist et al. used an electron beam to locally inactivate fibronectin, leading to cell adhesion only in unexposed areas [306].

4.1.3. Scanning probe methods

Dip-pen nanolithography (DPN) [280,307,308] and other atomic force microscope (AFM) based techniques such as nanoshaving of SAMs [280] offer high resolution and arbitrary patterning under mild conditions. These methods have been used in many studies for patterning proteins and peptides, including in studies aiming to stimulate receptor signaling. Typically this technique is used on SAM-coated surfaces, as the technique could be difficult to employ on non-flat surfaces. An indirect patterning method may be used, where alkanethiols with appropriate functional groups are patterned and the remaining space is backfilled with PEG functionalized thiols [228,229,309]. With this approach, resolution down to 100 nm has been achieved [309]. Direct patterning of proteins has also been demonstrated. Wilson et al. directly patterned thiolated collagen onto a gold substrate with 40 nm resolution [310]. While DPN has been shown to have a general resolution down to 10 nm, this resolution has yet to be demonstrated in direct DPN with proteins or other ligands [307], although it has been achieved using the nanoshaving technique [280,311]. There is also potential to independently pattern multiple ligands using this technique. Sekula et al. describe a system for DPN co-patterning of biotin and Ni-NTA moieties, and subsequent stability of an antibody pattern under cell culture conditions, which could seemingly be adapted for ligand presentation [312]. Throughput with a single tip is very low, however this can be increased using multitip systems [307]. One review of the area also notes that optimization of these systems can be time consuming [274].

4.1.4. Techniques utilizing self-assembly of nanospheres on a surface

Colloidal/nanosphere lithography techniques have been used by several groups for general protein patterning. Suspensions of essentially monodisperse colloidal particles are used as a mask, allowing the surface to be modified in those areas not obscured. Sutherland et al. used the technique to create fibronectin spots of between 100 and 1000 nm to examine integrin clustering [313–315], while a related technique was used to form triangular functionalized areas between the spheres [316]. Large areas can be patterned simultaneously using this technique, however, as can be seen in Fig. 7, spacing between the functional domains can be irregular.

Spatz and coworkers developed a method, termed “block copolymer micelle nanolithography,” using gold nanoparticles (GNPs) to control spacing between individual receptor–ligand complexes. GNPs were encapsulated within a diblock polymer micelle of controlled size, and these were allowed to pack into hexagonal arrays onto a surface. The polymer was then removed, leaving a pattern of GNPs with a consistent spacing, as seen in Fig. 8 [264,266]. GNPs are functionalized with ligands using metal–thiol bonds. GNP size was kept small enough to prevent more than one receptor interacting with each particle. The regularity of patterning and the ability to localize clusters of ligands with sub-receptor sized resolution (8 nm [264]) makes this a powerful method. Several later studies have led to important insights into links between individual receptor spacing and attachment events such as focal contact formation or tension within bound integrins [109,265,267,268].

4.1.5. Block copolymers and polymer blends

Blended polymer systems have been used to adjust the average spacing between ligands [29,317–320], where one component of the system is functionalized with ligand, while the other is left inert. Variable numbers of ligands per functionalized component allow overall density and clustering to be independently controlled. In an alternative approach, block copolymers can self-assemble to present nanodomains of functionalizable polymer on a low-fouling background (see Fig. 9) [321,322].

As “bottom-up” methods, these systems may have more variable spacing between ligands than “top-down” methods. However these materials do not require specialized equipment, are much simpler to implement, and can create patterns at higher resolution than many top-down approaches. Methods for increasing the regularity of these patterns would be of use, and considerations of any surface mobility of domains in these materials should be taken into account.

4.2. Three-dimensional patterning

Few methods have yet been devised that allow the patterning of ligands within 3D environments at the resolutions being examined here. One recent approach uses two photon irradiation to initiate covalent attachment with 3D spatial control, and examples of chemistries used with this technique are highlighted in Fig. 6. Resolution down

Fig. 7. Patterning via colloidal lithography. Colloidal particles of varying sizes (100–1000 nm) are deposited on a surface and used as a mask to create controlled-size patches of material that were later functionalised with adhesion ligands. [314]. Copyright 2010. Reproduced with permission from the American Chemical Society.

to 1 μm can be achieved [263] depending on the equipment used and plane of interest (resolution in the x – y plane can be an order of magnitude better than in the z plane). While the process is labor-intensive, the ability to create arbitrary patterns of ligands within a three-dimensional scaffold could enable many interesting studies of ligand patterning and cell behavior.

Several different groups have published patterning methods using this technique. The Shoichet group used two photon UV light to deprotect coumarin-caged thiols, which were then reacted with maleimide-containing proteins in three dimensions [211]. Following the initial deprotection, reaction of thiols with maleimide-barnase, and subsequent quenching, the process was repeated to pattern a second protein, maleimide–streptavidin. Streptavidin and barnase proteins were then used to attach biotin- and barstar-containing ligands via affinity interactions, creating patterns of two different signals. Anseth and coworkers used thiol-ene chemistry in several studies to pattern an RGD peptide in 3D within an independently photodegradable gel [71,219,263]. Using appropriately designed RGD ligand, containing both a cysteine for thiol-ene attachment and an *o*-nitrobenzyl ether photo-labile group for cleavage, they were able to show spatiotemporally controlled attachment and then release of the RGD ligand [263]. The West group used two-photon activation to react acrylated peptides with free acrylates within a PEG

hydrogel to achieve 3D patterning [271–273]. This method was also adapted to create patterns comprising more than one peptide [234].

Otherwise, top-down patterning in 3D is currently limited to lower resolutions (tens to hundreds of microns) than those discussed here, although technologies such as 3D printing are approaching relevant resolutions [323]. Alternatively, bottom-up strategies that create clusters of ligands within 3D hydrogels may be useful [324].

4.3. Spatially controlled co-presentation of different ligands

Having two binding motifs within close proximity may be desired, such as in the well-known case of PHSRN and RGD, two small peptide sequences within (respectively) the 9th and 10th type III domains of fibronectin. Co-presenting PHSRN with RGD has been found to increase affinity of RGD for $\alpha_5\beta_1$ integrins compared to $\alpha_v\beta_3$, and hence PHSRN is occasionally known as the “synergy” sequence [325]. For this interaction, PHSRN and RGD must be presented close enough to each other to bind to the same receptor, and may also need to be presented in a particular relative orientation [2].

Several different techniques have been used to co-present these two peptide ligands, while avoiding the complications associated with the myriad of active sites

Fig. 8. Arrays of consistently spaced RGD-functionalized gold nanoparticles and subsequent cell adhesion. Closely spaced RGD ligands supported cell adhesion, while cells did not adhere above a threshold of 73 nm. Images show nanoparticles with average spacing of (a) 28 nm, (b) 58 nm, (c) 73 nm, and (d) 85 nm. Due to the size of the particle used, only (at most) a single integrin receptor should bind to each nanoparticle. [264], Copyright 2004. Reproduced with permission from John Wiley & Sons.

in whole fibronectin. The simplest of these involves independently presenting RGD and PHSRN peptides [71]. In this case, no synergistic effects on cells were reported, and considering that close spatial coupling seems to be

important, these may have been minimal. On the other hand, Ochsenhirt et al. co-presented GRGDSP and PHSRN peptides independently within a Langmuir–Blodgett film and observed increased cell spreading [325].

Fig. 9. AFM image of polystyrene-block-poly(ethylene oxide) copolymer domains which assemble due to phase separation in blends with polystyrene homopolymer. Dark spots show the presence of block copolymers containing maleimide functional groups that can later be conjugated to receptor ligands. Variable spacing of domains is achieved by altering blend composition. [321]. Copyright 2009. Reproduced with permission from Elsevier Ltd.

Fusion peptides that link the two motifs with polypeptide spacers have also been found in several studies to show increased cell adhesion [326–328]. These peptides can be produced using solid-phase peptide synthesis, with the use of polyglycine spacers allowing the distance between motifs to be controlled. However, orientation is potentially not controlled, and the peptide is likely very flexible. Petrie et al. did not find any increase in adhesion when using these types of spacers, and found that a fusion peptide of this type still bound through $\alpha_v\beta_3$ integrins rather than $\alpha_5\beta_1$ [22]. Further work may still be required to elucidate the interactions between these two binding sites when co-presented in a fusion peptide.

A final method of ligand co-presentation uses a protein fragment, such as the 9–10th [23,329] or 7–10th [22,142,206] type III domains of fibronectin, rather than short peptides. Consequently, this method requires recombinant production of the protein fragment, rather than the simpler solid-phase peptide synthesis, but is more likely to control both the spatial and orientational relationship between RGD and PHSRN and lead to higher $\alpha_5\beta_1$ integrin activation than the previous methods [22]. However, binding may be able to be improved even further; Martino et al. showed that initial MSC adhesion and $\alpha_5\beta_1$ specificity to the 9–10th fragment was increased by using a mutated version of the protein [23]. This increase in binding was purportedly caused by a stabilized protein structure in the mutated fragment. Both fragments showed increased specificity compared to whole fibronectin, which contains multiple binding epitopes.

Co-presentation may also be required for other, as yet unidentified, groups of ligands in order to produce a desired biological effect. Other studies have focussed on co-presentation of adhesion signals and growth factors, using similar methods to those discussed above [93,237,330]. For example, Elloumi et al. investigated the action of a fusion protein containing both EGF and RGD signals [330].

4.4. Temporal patterning

The *in vivo* cellular microenvironment is dynamic, and evolves through processes such as cellular deposition and enzymatic remodeling of ECM proteins [331–333]. In recent years the creation of cellular environments that have the ability to change over time has come to the

fore of materials research. A large proportion of research in this area has focused on creating degradable microenvironments, particularly through the inclusion of short degradable linkers in hydrogel matrices, in order to allow cell migration. In contrast, the controlled temporal variation of ligand presentation is a relatively underexplored area of research.

Ligands may be physically encapsulated within the bulk scaffold material or within micelles or core-shell structures. However, we will limit the focus of this section to materials displaying time-varying ligands that are accessible to cells, rather than releasing them from internal reservoirs. For cohesion, we also restrict ourselves to degradation mechanisms that exclusively affect the ligand, and do not affect the bulk of the substrate, even though in native ECM the two may be interlinked.

4.4.1. Passive release

The natural affinity of many growth factors for binding to ECM proteins and GAGs such as heparin is commonly used to attach ligands to substrates and facilitate their controlled release. This approach was summarized in some detail in Sections 3.2.2 and 3.3.3. Passive release generally leads to an exponentially decaying release over a period of days to weeks. Vulic and Shoichet described a system where this exponential release could be tuned using a two-component system [334]. In order to modulate the release rate, they used recombinant FGF that contained an additional binding domain, and two different peptides on the substrate with different affinities for that binding domain. The ratio of the two binding peptides controlled the release rate.

Several types of linking chemistry are also sensitive to hydrolysis. We are unaware of any studies where hydrolysable linkages have been deliberately used for controlled slow release of ligands from a cell culture substrate, although this approach has been used for drug release from soluble PEG [335].

4.4.2. Stimuli responsive spatiotemporal control: researcher-responsive changes

Photodegradable linkers have been used to release RGD adhesion peptides from surfaces. Kloxin et al. described a system using a photocleavable RGD-acrylate monomer to incorporate the RGD ligand within a PEG acrylate hydrogel [336]. RGD could then be cleaved from the hydrogel

in three dimensions and at controlled time points using UV irradiation, inactivating cell adhesion. DeForest and Anseth extended this, allowing for both spatially-controlled coupling and release [263]. This work used an RGD peptide with both thiol and *o*-nitrobenzyl functionalities within the spacer. The peptide was first photocoupled within a PEG hydrogel via a thiol-ene click reaction, and could then be photoreleased via the degradable *o*-nitrobenzyl ether group. del Campo and coworkers also describe a system that incorporated an *o*-nitrobenzyl-containing linker for spatially controlled RGD release [337]. Conversely, the same group described the use of a caged cyclic RGD ligand, which can be made accessible (rather than released) in the presence of cells through UV irradiation [338,339].

Auernheimer et al. used an alternative approach, linking RGD to a surface via azobenzene-based spacers [340]. These spacer molecules undergo photoisomerization (changing between the *cis*- and *trans*- forms of the molecule) that leads to fully-reversible conformational changes within the molecules. The presence or absence of photostimulation led to reversible changes in spacer length and the subsequent availability of RGD ligands to cells for attachment. Previous studies have also described the use of azobenzene groups within the structure (rather than the spacer) of cyclic RGD peptides to create photoswitchable RGD ligands, although these ligands were used in solution rather than for cell-surface adhesion studies [341,342].

Other systems have used the addition of enzymes [343], free coil domains [344], or electrochemical stimulation [345] to temporally control ligand presentation from substrates. These lack the spatial control of photoresponsive systems, although they may be able to be combined with photoactivatable chemistries [345].

4.4.3. Stimuli responsive spatiotemporal control: cell-responsive changes

Matrix metalloproteinases (MMPs) and plasmins are proteases released by cells and found within the extracellular environment. Peptide sequences that are sensitive to protease degradation are commonly used as crosslinkers in hydrogel systems; these crosslinkers are degraded during culture, reducing the modulus and mesh size of the hydrogels and allowing cells to proliferate and migrate [346]. However there is also scope to use these sequences within advanced spacer molecules to allow release without affecting gel structure [347].

Salinas and Anseth compared the effects of MMP-13-cleavable RGD versus uncleavable RGD on chondrogenic differentiation human mesenchymal stem cells in PEG hydrogels [348]. While integrin signaling was needed to initiate differentiation, the authors showed that late-stage differentiation was increased when the RGD ligands were cleaved (via cell-secreted MMP-13) at an intermediate point of development. Another study used an RGD-degradable sequence fusion peptide as the crosslinker within a heparin/starPEG hydrogel [349]. Although RGD is not completely released within this system, degradation of the crosslinker may result in a change in accessibility of the RGD ligand, as well as reducing the modulus of the gel.

Otherwise, efforts at release have typically been targeted at growth factors, which often have short half-lives

in vivo [350]. Zisch et al. showed that certain forms of VEGF contain plasmin-degradable sequences, which could be used to release covalently-immobilized VEGF [193]. Hubbell and coworkers created several different fusion proteins, containing various growth factors along with degradable sequences [351–353]. MMP-cleavable linkers have also been used to attach model proteins [233].

Cells may also modulate their own environment by releasing and rearranging ECM molecules [354]. A recent study aimed to create surfaces where ECM proteins could be rearranged by cells during culture using lectin-mediated binding, a novel method of protein attachment, that mimics in vivo ECM processes [269]. Lectins are proteins that bind to particular saccharide molecules, many of which are present in ECM glycoproteins and also on the outside of the cell membrane. This study used lectins as crosslinkers, to non-covalently bind ECM proteins to a pattern of surface-bound polysaccharides. They showed evidence of the rearrangement of fibronectin by fibroblasts, but did not examine other proteins; however, providing a well-controlled initial environment while allowing cells to dynamically remodel at later time points is an intriguing goal.

5. Quantification of surface-bound ligand

In order to investigate quantitative relationships between ligand presentation and subsequent cell activity, accurate characterization of ligand-functionalized substrates is vital. Many techniques, summarized below, are available to quantify ligand concentration. However, as has been discussed, ligand activity can change markedly upon attachment to a substrate. Quantification of the bioactivity of attached ligands is often neglected, and (as discussed in Section 5.2) is an area where further development would be welcome.

5.1. Ligand density

Techniques for determining ligand concentration are discussed in this section, including sensitivity limitations, measurement of patterns and heterogeneities, and practical issues such as simplicity and cost. A range of analysis methods, along with their respective advantages and disadvantages, is presented in Table 7.

Many hydrogel matrices can be created in such a way that their ligand content is defined, using protein, peptide and synthetic hydrogels. Hydrogels made from ECM proteins are easily quantified at a basic level (e.g., 2 mg/ml collagen), but many of the protein–cell interactions are not well defined. Short peptides, modified proteins or pre-modified synthetic polymers allow more rigorous quantification of the signals being received by cells, and can be characterized using standard methods such as nuclear magnetic resonance spectroscopy or mass spectrometry. Accurate and simple control of ligand density is an advantage of these systems, assuming that the ligands are incorporated quantitatively as expected; however we will limit the focus of this section to quantification

Table 7

Summary of methods used for quantification of ligand density.

Method	Advantages	Disadvantages
Label-free, non-specific		
Colorimetric assays	Simple. Inexpensive.	Lower sensitivity and precision.
QCM/SPR	Can monitor over time. SPR in particular can be very sensitive, particularly for large proteins.	Require specialised surface, model 2D surfaces only.
XPS	Good surface specificity, typically <10 nm on polymer substrates. Fluorine tagged ligands can be used in XPS for specificity and increased sensitivity.	Detection via nitrogen abundance has low sensitivity. Modeling is required to calculate surface density. Ultra high vacuum required. Expensive.
IR spectroscopy	Simpler and less expensive than XPS.	Poor surface specificity, lacks ability to quantify surface density accurately.
UV–vis spectroscopy	Good sensitivity and ability to quantify surface density.	Rarely used technique, may require labeling.
ToF SIMS	Spatial mapping of ligands without a fluorescent tag. Very high surface specificity, which can be used to detect protein orientation.	Difficult to use for density quantification.
Label-free, ligand specific		
ELISA	Ligand specific detection without pre-labeling. Kits available. May be able to measure bioactivity to some degree.	Antibodies must be available and of high quality. Sensitivity is dependent on antibody activity and so requires careful calibration.
Soluble receptors	Probes ligand affinity and availability. May be able to detect changes in orientation/conformation of attached ligands.	Rarely used technique. Requires a further detection step.
Amino acid digestion	Non-specific method that still gives an indication of ligand type.	Requires acid solution for digestion step. Less sensitive than other techniques. Only used on hydrogel substrates at this point.
Labeled ligands		
Fluorescence	Simple visualisation of spatial distribution. Very commonly used. Protocols using releasable tags can avoid problems with quenching effects.	Fluorescence may be partially quenched by surface interactions. Protocols using releasable tags require more involved protocols. Requires calibration.
Radioactive tag	Very high sensitivity (fmol/cm ²). Some ability to examine spatial variations.	Requires specialised equipment and safety protocols.

of ligands after they are immobilized on/within a substrate at an unknown density.

5.1.1. Non-specific methods

Non-ligand-specific methods such as colorimetric assays, resonance methods and spectroscopic methods can all be used to quantify the attachment of ligands. These rarely require a change in protocol for different ligands (although they may require new standards), but similarly are not generally able to distinguish between different ligands.

Colorimetric assays are inexpensive and simple, but may be unsuitable for accurate quantification at low densities or for small peptides. Several of these techniques are reviewed by Noble and Bailey [355]. Goddard et al. notes several advantages of the BCA (bicinchoninic acid) technique over other similar protein assays, which include increased sensitivity and ability to be used directly on a surface [43]. The Iwata group used a microBCA assay kit to directly determine protein concentrations on a surface down to 300 ng/cm², with uncertainties in the order of 100 ng/cm² [207,209]. Boucher et al. also used the BCA assay to detect a modified EGF ligand down to 120 ng/cm², with variations of approximately ± 60 ng/cm², by comparing the concentration of ligand in the conjugation solution before and after the reaction [38]; however indirect quantification methods have many drawbacks including lowered reliability compared to direct techniques. In particular, ligands may loosely adsorb to the surface during conjugation, but then removed during subsequent washing, leading to an overestimate of attached

ligand density. Furthermore, published protocols have used bovine serum albumin rather than the protein of interest as a calibration standard, which could decrease the accuracy of the assay.

Surface plasmon resonance (SPR) and quartz crystal microbalance techniques (QCM) both rely on detecting resonance effects of deposited material. Unlike other methods, they can be used to monitor deposition kinetics continuously in real time. Each technique requires its own specialized substrate. Although these substrates can be modified, for example by spin coating a thin layer of polymer, this requirement can limit their usefulness outside of model systems.

QCM uses changes in the resonant frequency of a piezoelectric crystal to monitor mass that is either adsorbed or coupled to the surface. Commonly it also monitors energy dissipation at the resonant frequency, which can be related to viscoelastic properties of the surface. QCM can easily detect frequency changes that correspond to masses down to 10 ng/cm², and a resolution down to 2 ng/cm² may be obtainable in some systems [356]. While some modeling may be required (due to coupled water at the surface or within a polymer layer), the techniques for this are relatively well established. However, besides the adsorption of large extracellular matrix proteins, QCM is rarely used for quantifying ligand attachment to surfaces. More often, it is used to monitor protein adsorption processes, particularly in low-fouling materials [313,357].

SPR is more sensitive than QCM, with a lower detection limit of approximately 0.1 ng/cm², although the method of measurement is quite different [358]. This technique

examines the propagation of electromagnetic waves across a gold substrate, which is stimulated by an incident beam of light. At different angles of incidence, light is absorbed or reflected at different ratios, with a reflection minimum at a resonant angle. The buildup of thin layers causes a detectable change in this resonant angle, and with the application of certain assumptions as to this relationship, mass deposition can be quantified [359]. SPR is more commonly used for protein attachment quantification than QCM. In particular, the ease with which SPR can be used to investigate binding kinetics has made it particularly popular for examining affinity-based attachment (and release) of ligands [37,155,170]. One study used SPR to examine the interaction between soluble integrin receptors (as discussed in Section 5.1.2) and surfaces that contained a synthetic integrin ligand bound to a surface, and in particular their specificity to $\alpha_v\beta_3$ integrins over other types [33]. Another study quantified the amount of immobilized heparin on a substrate, and subsequently the amount of VEGF that bound to that heparin layer. In that study, 0.1 ng/cm² of VEGF was detected, close to the lower limit of resolution for this technique [52].

Spectroscopic methods such as X-ray photoelectron spectroscopy (XPS), infrared (IR) spectroscopy, UV–vis spectroscopy and mass spectrometry can also be used for ligand detection on a surface. These techniques can detect characteristic signals from components of a ligand and the material to which it is attached to, e.g., the elemental composition, functional groups, and parent ions or fractions thereof produced by ionization. These techniques are often unable to distinguish components of the attached ligand from similar components on the surface (e.g., peptide amides on an amide-containing surface), which may make some techniques of limited use in some situations. Unlike resonance methods, they are not restricted to specialized surfaces. For example, both XPS and IR spectroscopy have been used to characterize electrospun fibers [360].

The XPS technique measures atomic composition of surfaces, by analysing photoelectrons that are emitted when the sample is irradiated with (usually monochromatic) X-rays. The kinetic energies of the photoelectrons are characteristic for different elements and can also be used to discern certain functional groups via curve fitting of high-resolution spectra (particularly of the C 1s signal). Ultra high vacuum conditions are required, and typically photoelectrons are collected from the top 6–10 nm of a surface. Detection limits and precision can vary between the different elements, but are generally between 0.5% of atomic composition (for elements detected with low sensitivity, such as nitrogen) down to 0.05% for elements with more intense photoelectron peaks (e.g., many metals).

The signal from nitrogen may be used to detect the presence of protein and peptide ligands [11,361], although in practice this approach can be problematic; signals from nitrogen atoms are relatively weak, and it is often (but not always [254]) impossible to distinguish the signal from other forms of nitrogen present on the surface. Alternatively, other elements such as fluorine can be introduced into the molecules of interest. Fluorine-containing molecules are highly suitable for derivatization XPS because firstly, fluorine is an element not commonly

present on many surfaces, and secondly, the F 1s molecular orbital has a high photoionization cross-section, thus improving the sensitivity of XPS. This method may be particularly useful in cases where small ligands (with a high percentage of fluorine) are conjugated at high densities, but requires a modified ligand to introduce the fluorine [32]. Converting atomic abundance to an areal density is not generally attempted, however, with the use of several assumptions and modeling, atomic percentage of an element can be converted into surface density, with resolution down to tens of pmol/cm² [32].

IR spectroscopy can also be applied to the problem in a similar way to XPS, although to our knowledge it has only been used to detect (not quantify) ligand attachment [157,254,255]. The technique is sensitive to functional groups such as amides, which are present in proteins and peptides on a surface. It has relatively deep surface penetration compared to XPS [43,360], which limits its usefulness. However the equipment is less expensive than XPS, and unlike XPS does not require the use of ultra high vacuum (UHV). This makes it a potentially useful method for quick confirmation that ligands are present.

Tugulu et al. use UV–vis spectroscopy along with a pyrene-functionalized RGD peptide to quantify surface density [255]. This method appears to have good sensitivity (down to 0.5 pmol/cm²), but required assumptions about the extinction coefficient as well as a chemically modified ligand.

Time-of-flight secondary ion mass spectrometry (ToF SIMS) is highly surface sensitive, but difficult to use for quantifying ligand density [362]. However, its ability to map 2D patterns of ligands (in this case of an RGD peptide) has been used [230]. SIMS has also been used to investigate protein orientation at a surface (using its surface penetration of only ~1 nm) [363,364], as well as the surface composition of complex ECM protein mixtures [365,366]. Complex mass spectra of protein-modified surfaces can be analyzed using Principle Component Analysis [243].

5.1.2. Ligand specific methods for unmodified (label-free) ligands

These methods can distinguish between different ligands, and do not require special modification of the ligand prior to attachment.

Enzyme-linked immunosorbent assays (ELISAs) can detect a specific ligand without using any pre-modification, using antibodies to that ligand. The sensitivity of the assay is dependent on the activity of the antibodies; very high sensitivity down to 0.1 ng/cm² has been reported [52], although typical resolution is not this good. This technique is commonly used with growth factors, as many kits and antibodies are commonly available. In addition, antibodies against RGD [7], larger ECM proteins [269], fluorescein [367] and His6 [269] have all been used for quantification of ligand density. ELISAs can be sensitive to ligand conformation [358], and may be of increased usefulness when the antibody-binding site and the receptor-binding site are the same.

ELISAs are commonly used to examine ligands in solution, and simple indirect protocols that compare the

concentration of a ligand solution before and after conjugation can be used to infer surface density [368]. However, as with colorimetric tests, indirect testing can be inaccurate. Improved methods include applying antibody solutions directly to a solid substrate [48,49,52,369], or digesting a hydrogel substrate and analysing the resulting solution [176]. Some protocols may require an assumption that surface-bound enzymes have identical activity to those in solution, which may reduce the reliability of ELISAs for accurate quantification [99]. If no appropriate antibodies are available for detection, proteins can also be expressed to include further functional sections for detection via ELISA [370].

Soluble receptor proteins, instead of antibodies, can be used in a similar way. This can directly confirm the ability of receptors to bind and probe the affinity of the substrate-bound ligand for the receptor. While this is unique among the techniques discussed here, it still does not confirm the ability to induce downstream signals – that is, whether the ligand is acting as a full agonist, partial agonist or antagonist. As with ELISA, binding of the receptors to the substrate requires a further detection step, for example using SPR [33] or fluorescence [371].

Both of these techniques may be able to give some indication of the bioactivity of the attached ligand, as binding of the antibody/receptor is dependent on conformation. However this may also be a drawback; binding in this case is therefore dependent on two factors, density and affinity/bioactivity. Potentially the techniques discussed here could be combined with conformation-independent techniques (e.g., as discussed in the preceding or following sections) to separate these effects, and at the same time create a measure of binding and activity for the immobilized ligand.

Peptide digestion using acid solutions can also be used for detection of protein and peptide ligands provided the surface area and ligand concentration assessed are sufficiently high [372–374]. The immobilized ligands are digested along with the gel substrate, and the type and quantity of various amino acids are then detected using high performance liquid chromatography (HPLC). This technique can confirm the amino acid makeup of the attached protein or peptide, giving some level of specificity, but at the same time uses a general protocol that in principle does not require modification for other amino acid-based ligands.

5.1.3. Ligand specific methods using modified ligands

This section describes the use of ligands that are labeled with tags for quantification prior to conjugation to the substrate. Labeling may be particularly appropriate for peptide ligands, as their small size can make them difficult to detect using label-free methods.

Use of radioactive ligands for density quantification has been the gold standard for many years due to the high accuracy and high sensitivity (fmol/cm²) of the techniques [375]. Peptides or proteins are generally radioiodinated (I₁₂₅) via a tyrosine residue [103,151,237,258]. Radioiodinated ligands are generally diluted with unmodified versions, and the assumption is made that the two couple to the surface in an identical fashion. Gradient patterning may

be examined by taking sections of substrate and reading these individually [376]. Alternatively, Chollet et al. used ³H-labeled lysine to quantify density using a micro-imager with reported spatial resolution of 15 μm [377], while Clémence et al. were able to visualize 20 μm stripes of a ¹⁴C-labeled YIGSR peptide [378]. Techniques using radiolabeling have good accuracy and precision, but require specialized equipment. This, along with the involved safety protocols for working with radiation in most countries, prevents this technique from becoming standard.

Fluorescent ligands are of particular use in visualizing spatial distributions of ligands. Ligand distribution in many 3D structures can be examined using confocal microscopy, with spatial resolution down to 1 μm. Quenching from interactions of the fluorescent tag with a substrate can be a problem however, and quantification data from fluorescent tags on a surface may be only semi-quantitative [379].

Cleavable fluorescent tags offer one way around the surface-quenching problem, and have been developed as a high-sensitivity alternative to radiolabeling. One method uses an enzymatically-cleavable fluorescein to avoid quenching effects [101,379]. This method is easy to perform and can detect peptide density down to at least 1 pmol/cm², but requires an enzymatically cleavable sequence to be included within the peptide during manufacture. Coad et al. describe an alternative fluorescence method utilizing europium-tagged proteins [357]. A commercial kit was used to label human serum albumin with an isocyanated europium chelate. This technique is simple, can be performed on protein or peptide ligands post-translation or post-synthesis, and has high sensitivity (down to femtomolar limits of detection).

As discussed in Section 5.1.1, ligands can also be modified so that they can be detected via spectroscopic analyses like XPS and UV–vis spectroscopy. Other potential methods could be the immobilization of ligands via a stimulus-responsive linker (see Section 4.4) or a degradable bond (e.g., disulfide bonds, see Section 3.2.4; or His6-Ni-NTA complexes, Section 3.3.3). However, to date we are not aware of these techniques being used for quantification of ligand density.

5.2. Ligand bioactivity

Quantifying the bioactivity of ligands is in general far more difficult than quantifying their density, and detailed characterization is often neglected. In principle, the bioactivity of the attached ligand can be demonstrated by the growth of cells on the modified substrate, and the presence of a change compared to an unmodified control. While this is a standard approach, it does not give any indication as to whether full or only partial biofunctionality has been retained. It is also impossible to determine whether all attached ligands are equally biofunctional, whether receptor activation is already saturated, or whether bioactivity of the ligand is modified by presentation on a surface (see Section 2.2). Similarly, comparisons between soluble ligands and attached ligands to investigate biofunctionality, while common [43], should be interpreted with caution.

Another common method is to modify the ligand with a spacer prior to attachment on the substrate, and then to

compare the bioactivity (in solution) of the modified and unmodified ligands [201]. This method will confirm that the spacer itself does not interfere with receptor binding. However, interactions between the ligand and the surface may still affect bioactivity once the ligand is attached.

An advanced fluorescence technique, Förster resonance energy transfer (FRET), can be used to examine distance changes between two fluorescent probes. It has been used to examine the binding strength between receptors and integrin binding peptides [118], folding of proteins that are attached to surfaces [55,56], and also to investigate the strength of affinity interactions used to immobilize ligands [165].

Direct evidence for receptor–ligand binding and subsequent activation can be obtained by investigating the activation of signaling pathways. Markers are chosen depending on the receptor being targeted. Assembly of focal adhesions is now a common marker in studies of integrin-active surfaces, although these do not specify which particular integrin receptors are activated. Examining the phosphorylation of targeted receptors [52] or proteins further downstream [109] via western blotting is a method that can be widely applied. These experiments provide valuable quantitative information, both as an output measure of stimulation and as an input variable for looking at downstream cellular behavior (spreading, proliferation, differentiation, etc.). However, they may be cell-type specific; currently there is no standard for measuring the bioactivity of a ligand independently of cell type.

5.3. Quantification conclusions

Quantification of surface-bound ligands requires the researcher to examine both surface/bulk ligand density and bioactivity. Many techniques are available to quantify ligand density on a substrate, each of which has advantages and disadvantages in different situations. It is more difficult to measure the bioactivity of ligands, and currently this can only be done as an average of all attached ligands. Therefore more advanced conjugation methods (keeping all ligands identically presented, using oriented attachment methods and non-interacting substrates) are desirable. It is important to check downstream activation of signaling cascades (e.g., receptor phosphorylation and protein expression) rather than just the final biological result (spreading or differentiation), as this can provide both extra validation of receptor stimulation and a standard with which to compare bulk cell responses.

6. Conclusions

Ligand-modified substrates often provide an excellent platform to examine fundamental biological questions, as well as having potential applications in many sub-fields of biotechnology. However a thorough knowledge of a ligand and receptor's biology is required for optimal use, and many factors that can affect receptor–ligand activation may need to be considered. In this review we have examined techniques that have been used to attach ligands of various receptors to polymer or polymer-coated substrates, as well as techniques used in related fields that could be

adapted. We have detailed the advantages and disadvantages of various methods in a way that is broadly applicable, rather than as a collection of isolated cases.

When using substrate-bound ligands, activation of cellular receptors is affected by both the intrinsic bioactivity of a given ligand for that receptor, and modulatory factors arising from the conjugation and surrounding environment. Because of this, new conjugation methods are constantly being devised that offer increased ease of use, consistency of immobilization and other advantages over those previously used. Accurate characterization of substrates also requires an appropriate choice of method, and methods are constantly being developed and improved to perform this characterization.

The advanced techniques detailed in this review offer many advantages over simpler protocols. At the same time, many applications will require only simpler systems – non-optimal use can be “good enough” for many studies. We hope this review has provided an overview of both the techniques that are available for ligand conjugation in polymeric biomaterials, and the situations in which these techniques are most required.

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