The importance and clinical relevance of surfaces in tissue culture

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ABSTRACT: Cell and tissue culture has evolved from the use of simple glassware for the propagation of cells and tissues into a comprehensive platform for interrogating complex biological systems, directing cell fate and deriving products with clinical and therapeutic value. However, despite significant advances, current *in vitro* culture approaches remain limited in their capacity to model the clinical/biological complexities of disease, in part at least due to the deficiencies of existing culture materials. The challenge is therefore to identify innovative materials-based solutions that have greater control over cells *in vitro*, while better representing biological systems *in vivo*. Such platforms would be suitable for biomarker discovery and tissue engineering applications. This review examines the development of tissue culture materials, advances in our understanding of cell-surface interactions and the application of this knowledge towards the development of new approaches for better examining biological events.

The ability to culture cells and tissues *in vitro* is a fundamental aspect of modern science. Established early in the twentieth century, notably through the work of Harrison R.G. of John Hopkins University[1], the ability to culture cells and tissues has markedly improved during the intervening period. The field has progressed from an ability to maintain and culture tissue for extended periods, through the discovery and establishment of immortal cell lines, to today, where tissue engineering is making considerable progress in the production of artificial tissues and organs *in vitro* [2-4]. Key to these successes have been advances in the culture surfaces on which cells and tissues are grown.

This review summarizes progress in the development of tissue culture materials, highlights current requirements and existing limitations for *in vitro* culture, and examines their relevance to clinical questions and our current understanding of tissue culture materials design. Although long-established, current culture materials may not always be appropriate for modelling *in vivo* conditions, and innovative strategies are therefore required in order to overcome existing limitations.

1.0 Current Issues with Tissue Culture:

Numerous articles have highlighted the drawbacks and limitations of current *in vitro* culture systems [5, 6]. Concerns revolve around deficiencies in the culture systems and the tissue they generate. Although the latter can be linked to the quality of the initial cellular material, contamination and/or poor maintenance of historical cell lines [6, 7], it can also result from deficiencies in the culture systems i.e. not all cell populations are amenable to *in vitro* culture. Problems are compounded once tissue enters *in vitro* culture, as derived populations are expected to maintain their *in vivo* relevance. However, cells naturally adapt to the local environment and

prolonged culture of immortalized cell lines results in a progressive divergence from the parental population [6, 8, 9]. Although loss or gain of abnormal cell characteristics is a generic problem, it is more acute for cells exhibiting 'plasticity' such as stem cells, and the long term maintenance of pluripotency is recognized as a significant issue for stem cell research [10]. Taken together, it remains difficult to interpret the results of *in vitro* studies in the context of the *in vivo* situation. A practical example of this is the high rate of attrition for therapeutics, with less than 10% of candidates identified becoming licensed drugs [11]. The current consensus is that the failure rate for promising medical developments is, in part at least, attributable to the difficulty in translation of biocompatibility, toxicity or dose-responses that have been identified *in vitro*, into the *in vivo* setting [12-14].

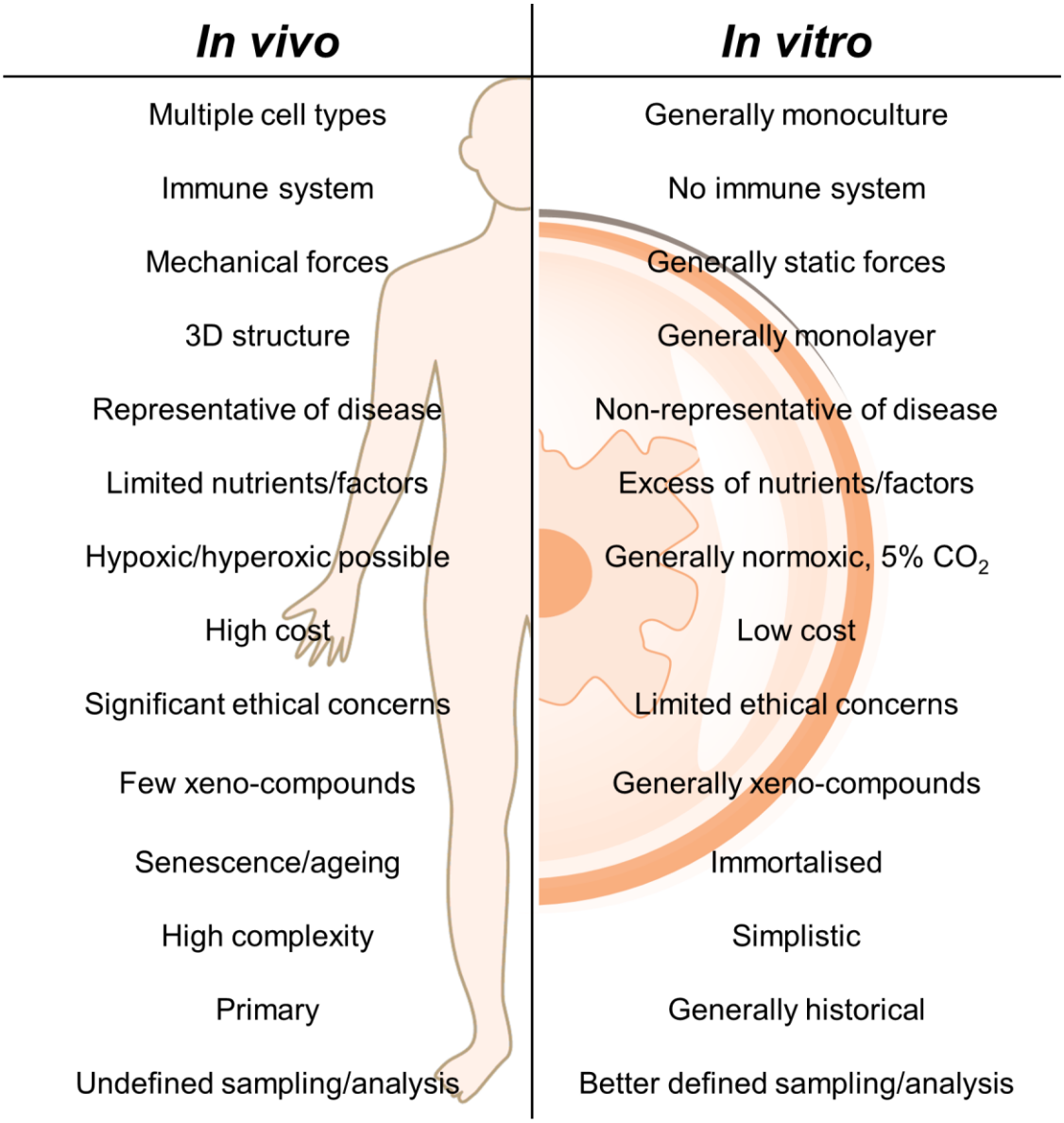

Figure 1. Comparing and contrasting the features of *in vitro* and *in vivo* tissue culture model systems.

Considering the problems, one might be prompted to favor *in vivo* model systems, Fig. 1. However, such systems are accompanied by ethical issues surrounding the use of animals, infrastructure, regulatory requirements and cost, and the data that they generate does not always reliably model or predict responses that occur in the clinical setting [15, 16]. *In vitro* models therefore remain an important mainstay of biological and clinical research, with the development

of novel *in vitro* models of *in vivo* biology having the potential to reduce the number of animals that are required for pre-clinical studies and therefore significantly impact on the application of the 3Rs (Replacement, Reduction and Refinement) principles [17].

2.0 Development of Tissue Culture Materials:

2.1 From Glassware to Plastics: Early tissue culture materials evolved from glassware that was available in the laboratory at the time. Although glass is an adequate surface for culture, most adherent cells require the support of an extra-cellular matrix for adhesion and survival and so glass often required modification with a range of different biological polymers such as agar, collagen, poly-L-lysine or cellulose in order to permit attachment and growth [18-20]. Many of the common matrix analogues or substitutes that have been used are summarized in Table 1.

Table 1. Matrix analogues and substitutes for tissue culture

Substrate	Role	Composition	Notes	Manufacturer	Reference
Cellulose	Adherence	β(1-4) linked D-glucose polysaccharide	-	Various	18
CELLstart™ & MaxGel™	Defined ECM homologue	Human origin ECM components	Xenobiotic free	Invitrogen & Sigma®	21
Collagen	Adherence, scaffold	Protein family from connective tissue, 29 forms identified	Matrix alternative, the hydrolysate gelatin derives from collagen	Various	19, 22
Entactins	Adherence	Glycoprotein family of the basement membrane	Contains RGD adhesion sequence	Various	23
Fibronectin	Adherence	~440 kDa ECM glycoprotein	Contains RGD adhesion sequence	Various	24
Foetal bovine serum	Adherence, proliferation	Complex undefined extract	High intra-batch variability	Various	25
Laminins	Adherence	Glycoprotein family of the basement membrane	-	Various	26
Matrigel™, Geltrex® & Cultrex®	ECM homologue	Biological extract of proteins with growth factors	Derived from Engelbreth-Holm-Swarm mouse sarcoma cells	BD Bioscience, Trevigen, Invitrogen	27
ε-Poly-L-lysine	Adherence, scaffold	Small (>20 units) natural homo-polypeptide of L-lysine	Bacteria derived, other homo-polypeptides exist	Various	20
Proteoglycans	Adherence	Family of heavily glycosylated ECM proteins	Grouped by glycosaminoglycan e.g. Agrin is a heparan sulphate	Various	28
StemAdhere™ & Vitronectin XF™	Adherence	Recombinant ECM protein	Xenobiotic free	Primorigen Biosciences Inc.	29
StemXVivo™ & Synthemax®	Defined ECM homologue	Recombinant ECM proteins	Xenobiotic free	R&D Systems, Corning®	29
Vitronectin	Adherence	~75 kDa ECM glycoprotein	Contains RGD adhesion sequence	Various	24

Abbreviations: ECM, extracellular matrix; RGD, Arginylglycylaspartic acid (RGD)

Today, glassware has been superseded by “plastic”, organic polymers, which are considerably cheaper and more versatile. The principal polymer used is tissue culture polystyrene (TCPS). TCPS differs from conventional polystyrene in that the surface of the polymer is modified to more readily permit cell attachment and proliferation. This is achieved *via* the introduction of a range of different chemical functionalities such as carboxyl, hydroxyl, ketone or formyl groups

to the surface using treatments such as sulphuric acid or oxygen plasma [30]. The nature of the functionality introduced influences surface performance in culture, an early observation was the preference in the hydroxyl component for BHK cell adhesion [31]. Surface treatment facilitates adhesion, and thus survival, by promoting the adsorption of extracellular matrix (ECM) components such as fibronectin and vitronectin from serum which is typically added to cell culture media, as well as the deposition of biomolecules that are endogenously produced during culture [31-34].

A wide range of culture materials and treatments have been developed since the introduction of TCPS, Fig. 2, examples are provided in Table 2. Advances in surface treatment techniques has allowed the production of surfaces with well-defined chemistries, such as the BD Purecoat™ series. This differs from conventional tissue culture plastic, in that surface functionality (be it amino or carboxyl) is of one type and tightly controlled [35].

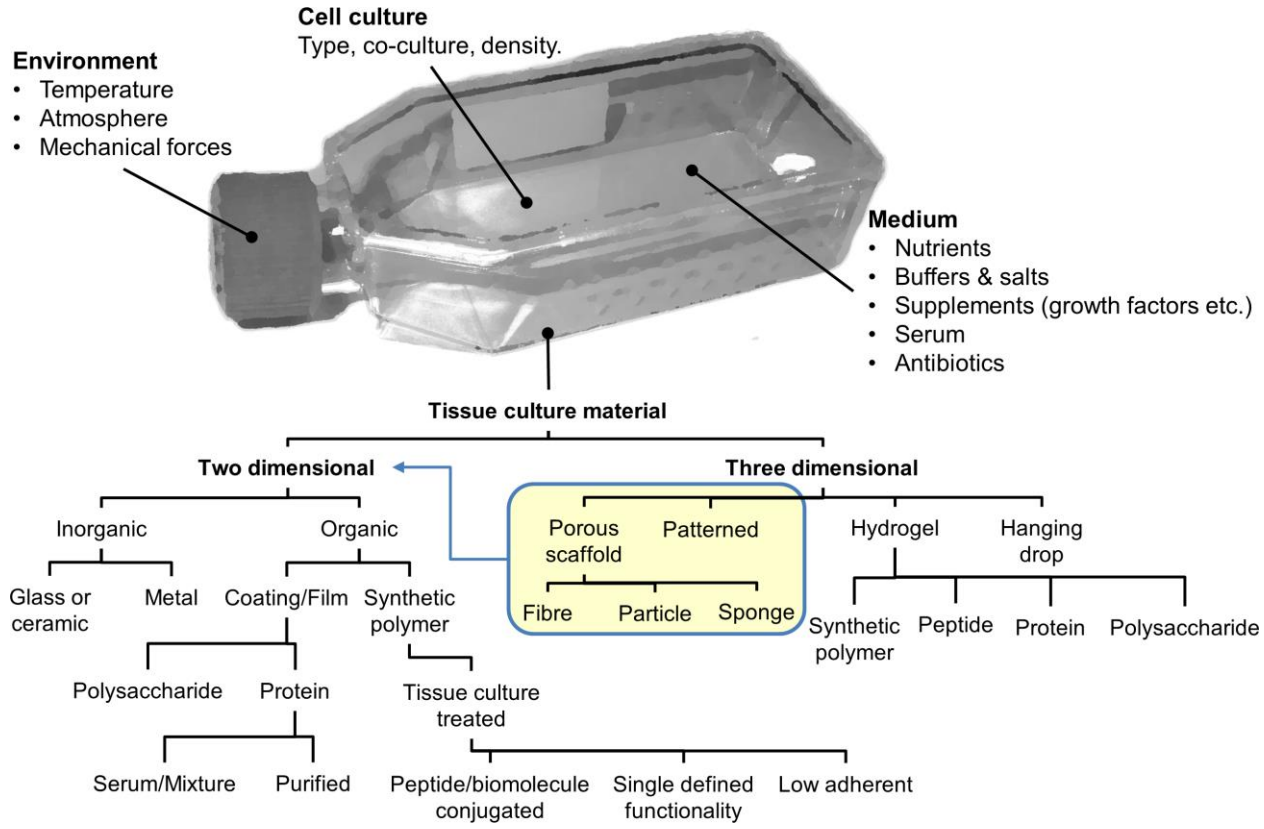

Figure 2. Different elements of *in vitro* culture systems. A scheme showing the different types of tissue culture material available is provided with examples given in Table 2.

Table 2. Tissue culture materials

Material	Role	Chemistry	Notes	Manufacturer	Reference
Agar	Adherent, 3D culture	Agarose and agaropectin polysaccharide	-	Various	18
AlgiMatrix®	3D tissue culture	Polysaccharide	Alginate based scaffold	Invitrogen	36
Alvetex® Scaffold	3D tissue culture	200 µm porous polystyrene membrane	Pore diameter is 40 µm with interconnects of 13µm	Reinnervate	36
BD PureCoat™	Adherent culture	Surface treated polystyrene	Single functionalities	BD Bioscience	35
BD PureCoat™ ECM Mimetic & Synthemax™	Adherent culture	Peptide conjugated polystyrene	Modified with synthetic peptides e.g. fibronectin and collagen I	BD Bioscience, Corning®	37
Corning® Osteo Assay Surface	Adherent culture	Patterned tissue culture polystyrene	Assess osteoclast & osteoblast functionality	Corning®	38
Glass	Adherent culture	Borosilicate glass	Acid treatment was common	Various	24
Hyaluronan	3D tissue culture	Polysaccharide of D-glucuronic acid and D-N-acetylglucosamine, >20 million Da	Hydrogels with differing chemistry such as growth factor release	Various	36
HydroMatrix™	3D tissue culture	Peptide hydrogel	-	Sigma®	39
Hydroxyapatite	Adherent culture	Calcium phosphate mineral	Indicates bioactivity	Various	40
Perfecta3D® & GravityPLUS™	3D tissue culture	None	Hanging drop for spheroid culture	3D Biomatrix, InSphero	41
Polyacrylamide	2D hydrogel	Polyacrylamide hydrogel	-	Various	42
Polycaprolactone	3D tissue culture	Polycaprolactone	Biodegradable	Various	36
Polyethylene-Glycol, QGel™	3D tissue culture	Polyethylene glycol hydrogel	Differing chemistry such as light sensitivity or biodegradable	Various, QGel	36
Polystyrene (TCPS)	Adherent culture	Surface treated polystyrene	Single or mixed surfaces chemistry	Various	31
TCPS low adherence	Low adherence	Hydrophilic, neutral charge	Corning® use hydrogel	Various	43

Changes in culture practice, such as the more widespread use of serum supplementation and the availability of new materials have, to some degree, eliminated the requirement for pre-treatment

with ECM analogues/substitutes such as collagen. However, challenging applications such as the culture of primary cell lines having a limited proliferative capacity and stem cells continue to require pre-treatment strategies [29]. Our increased understanding of cell adhesion and its requirements has increased the range of naturally-derived or recombinant proteins that can be used for the pre-treatment of culture surfaces (see Table 1).

The discovery of cell adhesion molecules (CAMs) and their role in cell-cell and cell-surface interactions has led to the development of surfaces incorporating the principals of cell adhesion and the ECM such as the BD PureCoat™ ECM Mimetic & Synthemax™ surfaces [44]. These are chemically functionalized with peptides derived from the active sites of proteins that are implicated in cell adhesion, such as the tripeptide arginylglycylaspartic acid (RGD) [45]. The RGD sequence is implicated in cellular attachment *via* integrins (such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$), [46] and can be used to coat synthetic scaffolds in order to enhance cellular attachment by mimicking *in vivo* conditions.

Patterning of surfaces using lithography, chemical or mechanical processes has also been used to yield better mimics of *in vivo* environments [47]. For example, the Corning® Osteo Assay Surface has been modified with a synthetic crystalline calcium carbonate coating in order to better resemble the surface of bone for assessing the performance of osteoclasts and osteoblasts [48].

2.2 Advanced Tissue Culture Concepts: Building on the principals of matrix substitutes and advanced surface chemistry, a range of advanced tissue culture concepts have emerged. Many advanced tissue culture systems have moved towards the presentation of an artificial ECM, initially *via* the presentation of biologically-derived ECM components, for example Matrigel™ [27]. More recently, substitutes that are artificial in nature (e.g. SemXVivo™) have been developed

and these benefit from being chemically defined and free from xeno (other species-related) components [29, 36].

Parallel to the development of xeno-free ECM mimics has been the introduction of serum-free (not necessarily protein- or peptide-free – although these also exist) media systems [49]. Serum is problematic in that it is poorly defined with batch-to-batch variations in its ability to sustain the growth of different cell populations and sub-populations. Although the concept of serum-free culture has existed for a long time, its implementation has been difficult. The removal / absence of serum components can trigger apoptosis of cells in culture. Although certain neuronal cell lines have been adapted to serum-free media, success with other lines remains elusive, not least due to the complexity of individual line requirements [50].

Although there have been significant advances in cell culture technologies, the adoption of advanced culture systems continues to be poor. For example, the Google Scholar search 'tissue culture "purecoat"' returned 61 entries in total, 'tissue culture "polystyrene"' returned 2,400 entries for the first five months of 2015 alone. This disparity can, perhaps, be understood by the niche application of specialist materials and the established nature of existing materials such as TCPS which offers acceptable performance for most applications. Although the issue of *in vitro* relevance could progressively be resolved by ever increasingly complex *in vitro* systems, such systems would be progressively more difficult to standardize and validate, and would likely be poorly adopted based on the uptake of even the modest advancements that are currently available (though these have not always had such well-defined applications or requirements).

2.3 Three Dimensional Culture Systems: The concept that the culture environment should closely replicate *in vivo* conditions now incorporates the view that tissue culture surfaces should

no longer be ‘flat’ surfaces. Although ‘3D’ materials have existed for some time (e.g. agar), considerable interest in the application of 3D tissue culture systems has developed and these platforms are now becoming more widely employed, with a multitude of different methods for achieving such materials having being developed (see Table 2 and Fig. 2) [51, 52]. Cells proliferate and migrate within and atop these 3D materials and those such as hydrogels can approximate biological structures such as the ECM while displaying ‘smart’ properties such as an ability to respond to culture conditions and external stimuli[53]. The interest in the field has generated many informative reviews of 3D culture systems [36, 54, 55], with an emphasis on hydrogels as mimics for extracellular matrix [56].

Relevant to our emphasis on surface chemistry is another example of 3D culture; spheroids. These require cells to culture independently of adhesion to the surface and the generation of which relies on low adherence culture materials.. These can be achieved by ‘passivation’ of a surface using, for example, a hydrophilic, neutrally charged hydro-gel layer that prevents protein uptake and cell adhesion. Other non-adherent systems use only the air/liquid interface which is formed through surface tension [41, 43]. Spheroid cultures are popular *in vitro* models due to their emphasis on cell-cell interactions and the secreted microenvironment, such approaches can also be used as a platform for generating complex 3D tissues [57].

A trend throughout the development of new tissue culture materials for applications ranging from tissue engineering to cancer research has been to apply our increasing understanding of the *in vivo* microenvironment to develop materials with local culture environments that are ever more representative of the *in vivo* conditions [58]. If the problem of *in vitro* relevance can partly be attributed to deficiencies such as existing culture systems not mimicking *in vivo* conditions, then it is unsurprising that, when conditions are modified to better reflect conditions *in vivo*, different

morphological, proliferative and dose responses to therapeutic drugs are noted [59]. As such, it is worth re-examining our conceptual understanding of a tissue culture surface.

3.0 Defining a Model of Cell Surface Interaction:

Tissue culture materials are all too often considered as merely a surface on which cells grow. Cell surface interactions can be difficult to visualize, as they encompass a complicated three dimensional arrangement and interaction of a multitude of different molecules and structures (both biological and non-biological) that operate on different length and time scales. A pictorial representation of cell-surface interactions and some of the processes involved is shown in Fig. 3A-F.

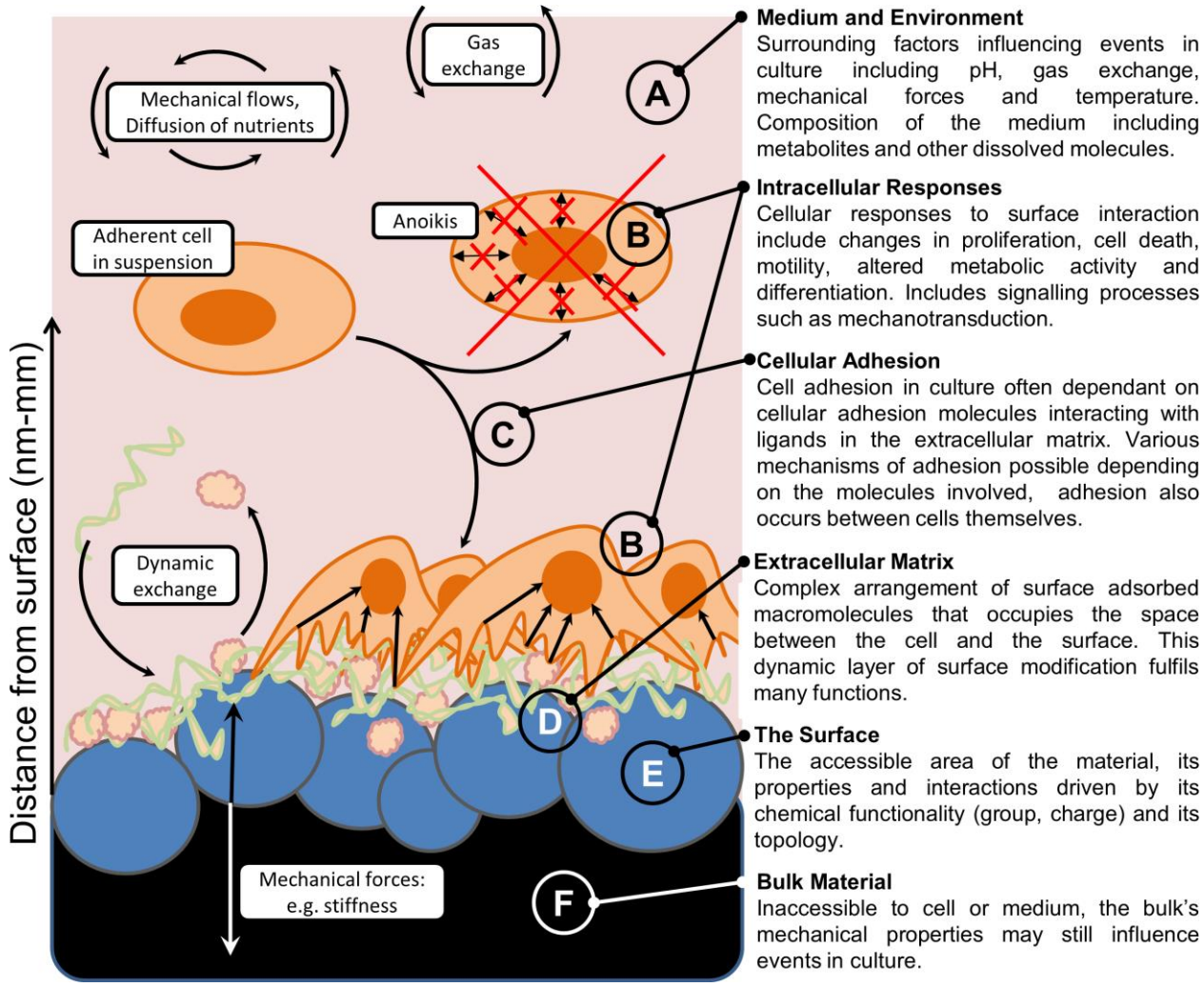

Figure 3. Model of adherent cell culture and representation of some of the factors and influences on the cell. The various elements represent; A: medium and surrounding environment, B: cell body and response, C: cell adhesion, D: protein adsorption (ECM), E: accessible surface, F: bulk substrate.

3.1 The Medium & the Environment (A): Cell surface interactions involve an extracellular component between cell and surface and intracellular components that are modified as a result of surface interaction; the cell's response. Events on the surface (such as protein adsorption) will be influenced to a large degree by the surrounding environment such as composition of the medium and environmental factors (temperature, pressure, CO_2 concentration etc.), Fig. 3A. Tissue culture media themselves are complex compositions of salts, metabolites and potentially dyes,

antibiotics as well and undefined mixtures of proteins, metabolites and signaling molecules of biological origin [26]. All of these elements contribute to the significant complexity of the culture surface under different conditions.

3.2 Intercellular Implications of Cell-Surface Interaction (B): The presence or lack of contact with surfaces (or rather surface adsorbed biomolecules) has a significant effect on a wide range of cell behaviors, including motility, morphology, proliferation and differentiation, Fig. 3B [60]. Motility, as a response to a stimulus or as a process in the pathology of disease and its spread (for example anoikis and metastasis) involves dynamic interactions of the cell cytoskeleton with the underlying extra-cellular matrix (ECM), permitting cell relocation [61, 62].

Adhesion is a matter of life and death for anchorage-dependent cell types, as cells unable to attach to the ECM undergo a form of programmed cell death called anoikis [63]. Without the formation of cytoskeletal elements associated with ECM adhesion, cell death is induced through caspase signal transduction and, when adhesion site components such as focal adhesion kinase are compromised, DNA synthesis is inhibited [64, 65]. Conversely, attachment promotes proliferation through cell division, with focal adhesion being implicated in many cell proliferation pathways such as the focal adhesion kinase and extracellular-signal-regulated kinase mediated pathways [66].

Cell surface interactions have been demonstrated to play an important role in cell fate, as they can modify the capacity of cells to undergo differentiation or maintain pluripotency [67, 68]. The mechanism of translating perturbations to the cytoskeleton into cell responses is known as ‘mechanotransduction’ [69], in which forces exerted on the cytoskeleton of the cell and changes in the availability of binding sites for cell adhesion molecules due to surface features influence the

biochemical pathways of the cell [70]. As an example, mechanical perturbation of cells can induce expression of the transcription factor Twist and differences in ECM stiffness are associated with the process of tissue differentiation [71].

Several biochemical pathways have been identified as having a role in mechanotransduction. The transcription coactivators YAP/TAZ have been implicated in sensing cytoskeletal tension, their activity is modulated by mechanical inputs, inputs which can be overruled if YAP/TAZ activity is manipulated artificially [72]. This activity is independent from many elements of the Hippo signaling pathway and instead depends on Rho GTPase activity and actinomyosin, though YAP/TAZ are associated with multiple inputs [72, 73]. Key sensory molecules in mechanotransduction include the cytoskeletal elements as noted above but also G-protein coupled receptors, growth factor receptors and stretch activated ion channels [71]. In addition to YAP/TAZ these sensory elements relay extracellular cues into pathways such as MAPK [71, 74] and P13K/Akt [75].

Mechanotransduction can be actively exploited to induce cell responses *via*, for example, controlling substrate stiffness [76], or topology [77]. The ability to precisely measure forces at the substrate, the plasma membrane and in the cytoskeleton is a significant aid in unravelling the influence that mechanical forces and their inducement have on cellular responses [78].

3.3 Cellular Adhesion (C): The next component of the model involves cellular interactions with extra-cellular molecules that have been adsorbed on to the cell surface - the process of cell adhesion, Fig. 3C. Protein adsorption is considered to be an essential pre-requisite for cell adhesion, with multiple components of the ECM playing a role. These include proteins such as collagen, vitronectin and fibronectin, all of which have been known to enhance cell adhesion to

surfaces for some considerable time [24]. However, certain proteins are more relevant than others, and their specificity is dependent on individual cell type. As an example, the adhesion and proliferation of bovine corneal and arterial cells is impaired in cultures lacking vitronectin [24].

Cell adhesion to surface adsorbed ECM proteins is facilitated by cell adhesion molecules (CAMs) such as the calcium-dependent class of adhesion molecules known as integrins [79]. The ECM contains an abundance of amino-acid motifs with pro-adhesion effects; the best characterized being the RGD sequence, a ligand for $\alpha_v\beta_3$ integrin receptors for example [67, 80]. The modification of surfaces to present this sequence (e.g. covalently grafted using N-hydroxysuccinimide) enhances focal adhesion and induces the differentiation of adherent cells such as osteoblasts [80-82]. Focal adhesion encompasses large and dynamic macromolecular assemblies comprising many individual integrin receptors and their associated proteins [61, 83].

Adhesion is not a guaranteed outcome of cell-protein-surface interaction, as stable attachment cannot occur when surfaces cannot support CAMs [84, 85]. Other cell adhesion processes such as anchoring, and the establishment of tight and gap junctions are associated with cell-cell rather than cell-surface adhesion [86]. In tissue culture design, cell-cell interactions can be just as important, or of greater importance, than cell surface interactions such as in spheroid formation using 3D culture and the study of cell aggregation and disaggregation processes like metastasis [57, 87].

3.4 Extracellular Matrix; Biological Surface Modification (D): Cell-protein interactions are, in part, dictated by the properties of the surface; its ability to absorb the ECM and the nature of the ECM supply in the culture environment e.g. supply of CAMs in the media [88, 89]. Cell responses such as surface adhesion and subsequent differentiation are the result of interactions

between the surface of the material and the macromolecules that adsorb to the surface, and are influenced by the surrounding environment or 'niche' [84, 89]. The nature of the ECM reflects the broad range of biomolecules such as proteoglycans, polysaccharides, fibrous proteins like collagens and adhesion proteins such as fibronectin and vitronectin that are available [90].

Macromolecules such as proteins and carbohydrates are important constituents of most biological media and will adsorb to the surface over time, displacing bound water, ions and other smaller species. This adsorption process starts almost immediately the material enters the biological environment [91]. The process is also dynamic, with the surface adsorbed layer being continually remodeled as conditions change, for example through the Vroman effect, a process by which fast binding protein species on the surface are exchanged with other species of higher surface affinity over time [92].

Physiologically, the ECM occupies the space between cells and is the primary constituent of connective tissue [93]. The role of the ECM is as diverse as its constituents, and its functions include, but are not limited to, acting as a shock absorber, scaffold, store of energy and signaling molecules and finally as a site for cell attachment [93]. In the laboratory, the adsorbed macromolecules act as a dynamic layer of surface modification, altering the chemistry and topology of the surface, with this modification being influenced by the properties of the surface itself (a Smart material!). Properties of the surface such as its topology can alter macromolecule loading and characteristics such as the secondary structure and orientation of proteins, which can vary from molecule to molecule, and surface to surface [94-96].

This dynamic environment and the formation of the ECM is heavily influenced by proteins that are provided as a consequence of serum supplementation, as well as those that are produced by

the cells themselves. This diverse mixture of proteins, metabolites and other molecules is generally poorly defined both initially and during culture.

3.5 The Surface; between the Bulk Material & Medium (E): The penultimate layer of the model is the surface itself (Fig. 3E). Although at its simplest, this can be described as being no more than the top-most layer of accessible atoms, exact definitions are difficult as it varies depending on the interacting molecule. For example, the covalent radius of a hydrogen atom is 25 pm, but for a sodium atom this is 180 pm [97]. As a consequence, the two atoms will describe a given surface differently. This holds true for larger molecules, in that the form of the surface, its topology and porosity (Fig. 3F) all affect how molecules will interact with the surface and contribute to the bulk surface properties such as wetting [98, 99]. Tissue culture materials have a wide variety of forms depending on the processing methods used, Table 2.

Whatever the 'surface' is considered to be, the intra-molecular interactions it promotes strongly affect the surface properties exhibited. In the case of tissue culture polystyrenes, the surface is comprised of hydrocarbon chains containing exposed phenyl groups and a mixture of various oxygen (e.g. hydroxyl or carboxyl) or nitrogen (e.g. amine) containing groups, and different processing can vary their relative composition [100]. The introduction of polar species to polystyrene permits hydrogen bonding and electrostatic interactions, both of which influence solvation. Depending on solvent composition and surface properties, other entities such as counter-ions may also be present. In the biological context, most surfaces adsorb a wide variety of biomolecules such as proteins from a media.

There has been a push in recent years to move away from 2D culture systems, and this has been prompted by strong arguments in terms of biological relevance [51, 59]. It is worth appreciating at

this point that the distinction between 2D and 3D materials becomes immaterial as the molecular scale decreases. This is not to disparage 3D materials, providing many 2D regions of interaction and arranging them in a defined (or undefined) manner around the cell clearly influences cell response [54, 59]. The mechanisms governing how surface properties influence protein adsorption and hence cell response in 2D materials should translate to 3D. The difficulty in precise characterization of surfaces in 3D e.g. molecular orientation within a gel, suggests that 2D materials still have an important role to play in the development of new tissue culture materials and that the rush for 3D 'effects' should not come at the expense of our fundamental understanding of the principals operating in 2D. A comprehensive understanding of these phenomena is currently missing and considerable work is required to show how surface properties (chemistry and topology) guide protein adsorption and as a result induce cellular responses.

4.0 Tissue Culture Surfaces as Tools to Control Cell Response:

Investigations of cell-protein-material responses have been performed using a wide range of materials and cell types, and a number of reviews concerning the responses of specific cell types such as neuronal [101] and antigen presenting cells have now been published [102].

4.1 The Influence of Topology: Early observations identified the ability of topological features to guide the morphological organization of cell populations in terms of alignment and elongation, a phenomenon known as 'contact guidance' [103]. Later studies expanded on the influence of topology on proliferation, adhesion and, as interest in cell lines important for tissue regeneration (e.g. transient stem cell populations) increased, topological features that confer control over cell

renewal and differentiation have been identified [104-106]. Fig.4 illustrates the scale at which different cellular and protein responses have been observed.

Topology has been explored in a controlled manner, principally by using organic polymer materials and techniques such as lithography for 2D and electrospinning for 3D materials, both of which allow fine control of the structure on the micron to nano scale [107]. A broad range of materials encompassing most major classes of plastic, including biodegradable polymers such as polycaprolactone, have now been examined [10]. Responses over scales of a few nanometers (surface roughness) to hundreds of microns have been explored, and the effect of feature shape, size, orientation and density within these range of scales, Fig. 4, have been considered. Mechanical properties such as stiffness have also been assessed [108].

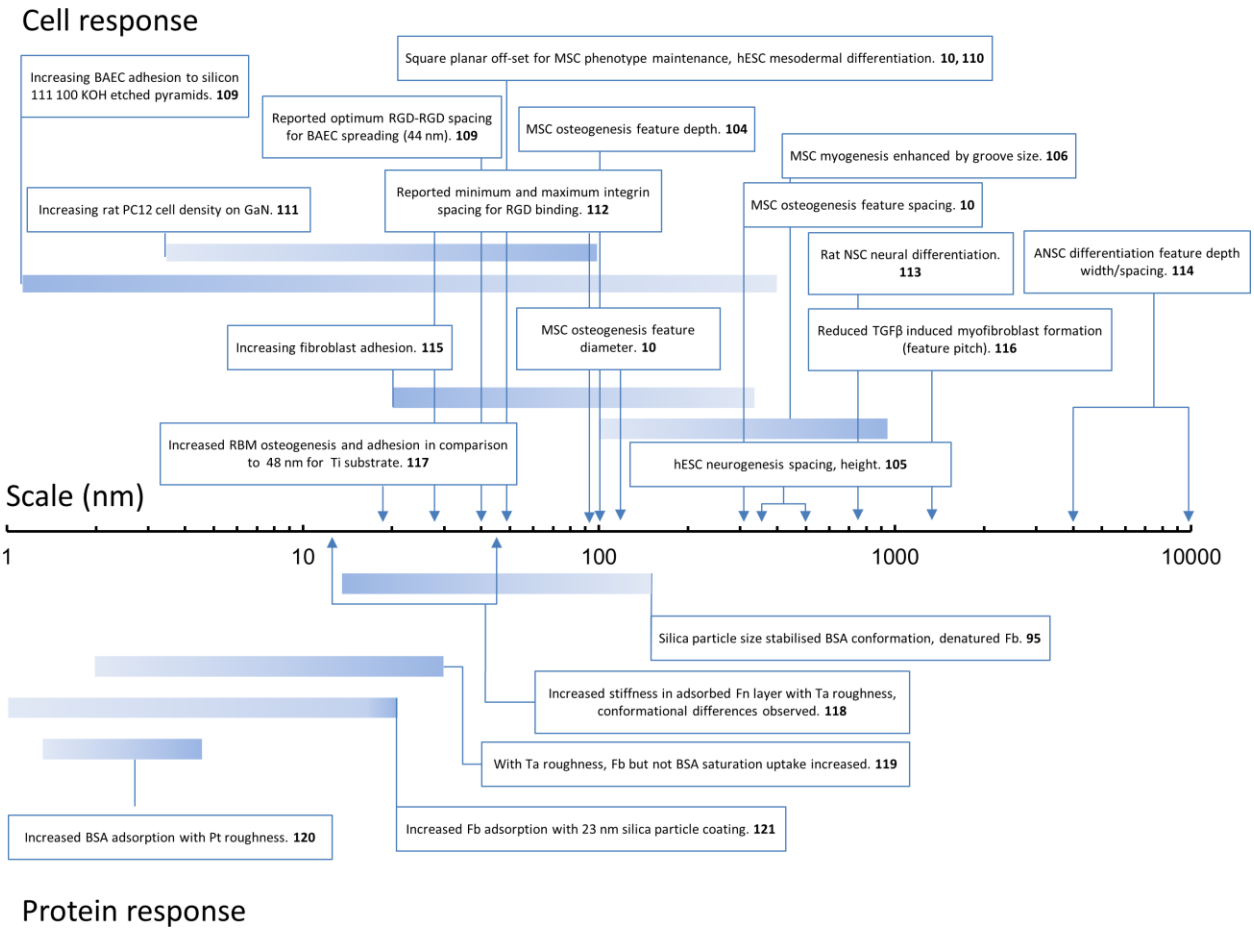

Figure 4. Influence of surface topological features at different scales on both the protein microenvironment and cell response. Bars with shaded gradients show cell and protein response across a range of topological sizes.

Above one micron, cellular responses are generally defined *via* contact guidance, with phenomena such as axon outgrowth processes noted at the 'smaller features' of the topology, e.g. edge effects [122]. As the scale of surface topological features (pits, islands, columns and gratings) decreases to the range of several hundred to several tens of nanometers, a plethora of cellular responses, across the full range of cell and material types (Fig. 4) is obtained, for example, cell-surface adhesion increases [106, 109, 115]. Studies on a variety of cell lines and surfaces have shown that neurite outgrowth in the presence of nerve growth factor is enhanced by channels 70-250 nm in width with a fixed depth of 300-600 nm [122, 123]. However, cell responses to topology are not always linear, and may be cell type-dependent (e.g. fibroblasts vs. mesenchymal cells) [104].

For 'plastic' populations such as mesenchymal stem cells (MSCs), differentiation effects such as osteogenesis or neurogenesis have been observed across the scale [105, 111, 114]. Feature size dependent effects have been observed, such as rat neural stem cell oligodendrocyte differentiation, proliferation and aggregation being favored for smaller electrospun polysulphone fibers (range examined was 283-1452 nm Ø), whereas intermediate (~749 nm) or larger fibers favor neuronal differentiation [113]. Alignment and differentiation (adipogenesis and myogenesis) of rat MSCs has been shown to be influenced by grooved surfaces (width/depth of 450/100, 450/350, 900/100 and 900/550 nm), the latter in a groove size dependent manner [106]. Fewer observations have been reported for experiments performed using topological features smaller than 20 nanometers (Fig, 4), and these suggest that the ability of cells to sense topological changes at this scale becomes more limited [112, 115]. However, at this lower end of the topology

length scale, topological features are known to drive increased adsorption and deformation in the protein microenvironment, Fig. 4 [119-121].

Studies on single size features within the range of one micron to tens of nanometers, have identified specific cell responses such as the transition of embryonic cells to a neuronal phenotype in the absence of traditional chemical agents being induced by polyurethane acrylate ridges/grooves of 350 nm spacing and 500 nm height [105]. In addition to inducing differentiation, the maintenance of MSCs for extended periods has been demonstrated using 150 nm polycaprolactone pits with a 300 nm square planar spacing. However, and in contrast, pits offset from a square planar arrangement by 50 nm enhance osteogenesis [10]. The apparent importance of these relatively discrete changes in topology on cell response highlight the complexity of topology-related responses, as well as the difficulty in interpreting a multitude of experimental observations that are taken under very disparate conditions.

4.2 The Influence of Surface Chemistry: The influence of chemical functionality has been primarily explored using self-assembled monolayers (SAM), as this approach can achieve a uniform loading of the desired functionality [96, 124]. Fig.4 illustrates the relationship between water contact angle and cellular and protein responses that have been observed for a number of surface chemistry studies. A range of functionalities have been studied (Fig. 5, Table 4) and, similarly to topological studies, work has focused on cells of a plastic nature that are applicable to tissue engineering.

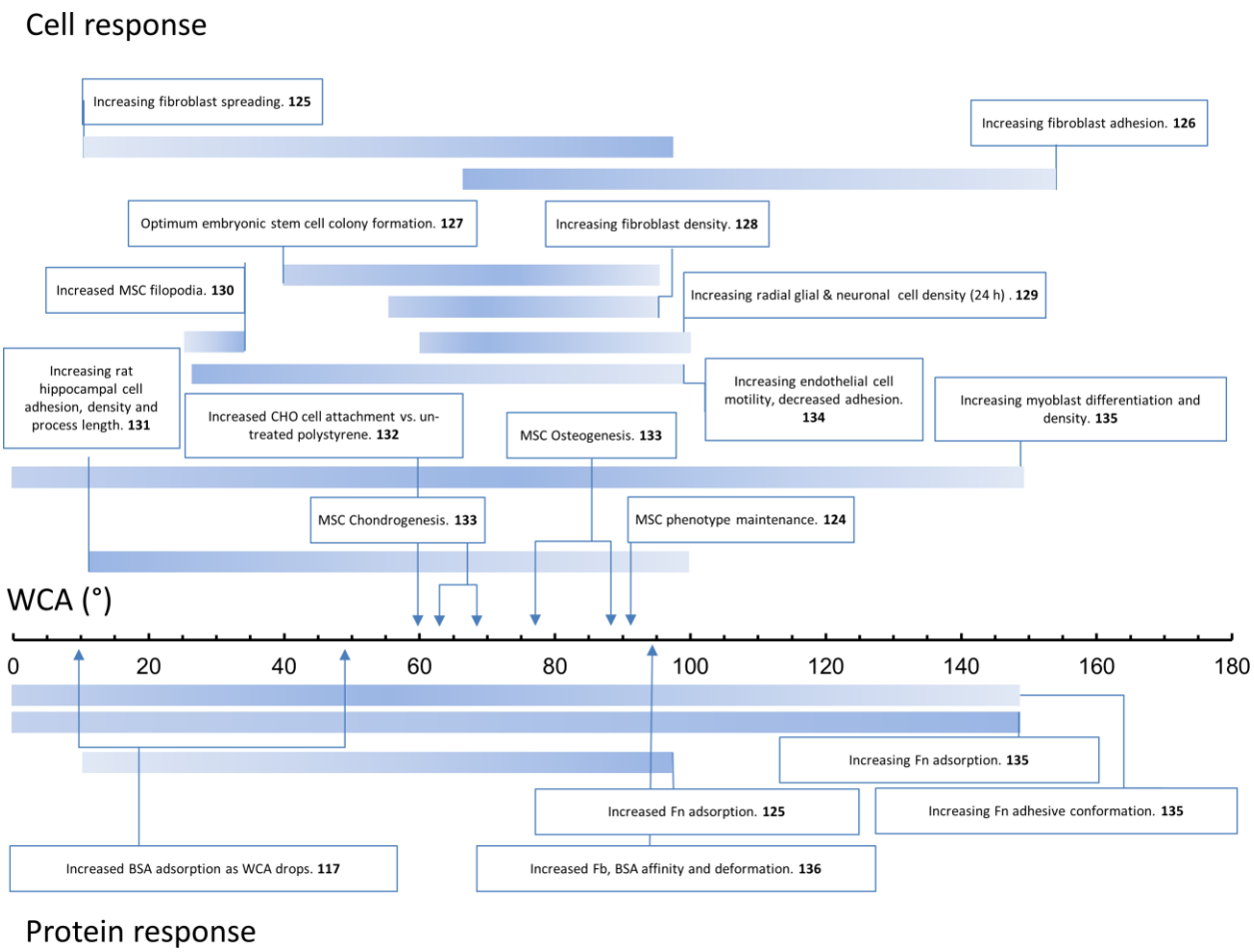

Figure 5. Influence of water contact angle (WCA) for a range of chemically distinct materials on both the protein microenvironment and cell response. Bars with shaded gradients show cell and protein responses across a range of WCAs.

Table 4. Influence of surface chemistry on the protein microenvironment and cell response.

Functionality	Properties	Protein response	Cell response
Carboxyl	Polar, acidic	Fn binding of both α5β1 and αVβ3. [96]	Chondrogenesis. [122]
Phosphate	Polar, acidic	-	Osteogenesis. [137]
Silanol	Polar	Increased BSA binding, deformation *vs.* CH_3. [95]	-
Hydroxyl	Polar	Fn selective binding of α5β1 integrin. [96] Increased BSA binding, deformation *vs.* CH_3. [136]	Osteoblastic differentiation. [96, 138] Chondrogenesis. [133]
Sulphone	Polar	-	Fibrogenesis. [130]
Bromine	Polar	-	HASC Adipogenic differentiation. [139]
Amino	Polar, basic	Fn selective binding of α5β1 integrin. [96]	Osteoblastic differentiation. [96] hADSC osteogenesis, high growth. [139] MSC differentiation. [140] Mesenchymal population loss. [141] MSC osteogenesis. [133, 138] Adipogenesis. [138]
Thiol	Non-polar	-	Chondrogenesis. [139]
Silane	Non-polar	-	Osteogenesis. [124]
Methyl	Aliphatic	Increased Fb deformation *vs.* SiOH. [136]	MSC phenotype maintenance. [140] HASC low growth. [139]
t-Butyl	Aliphatic	-	Adipogenesis. [137]
Phenyl	Aromatic	-	Chondrogenesis. [139]

Early studies revealed how functional variations on model surfaces (-OH, $-NH_2$, -COOH, CH_3) influenced the adsorption kinetics of key proteins (e.g. fibronectin), and protein characteristics upon binding (e.g. availability of integrin binding sites due to fibronectin refolding on adsorption) [96]. Protein binding responses were related to subsequent cell responses (e.g. differentiation of immature osteoblasts) [96]. That surface chemistry modifies protein conformation has been further explored and, although differential responses continue to be observed, questions remain as to how initial surface chemistry sustains cell differentiation over extended periods [138]. For example, in a 3D PEG-hydrogel environment, MSCs have been shown to differentiate *via* adipogenic or osteogenic pathways on t-butyl and phosphate functionalized surfaces respectively [137]. Furthermore, poly(lactic-co-glycolic acid) and PEG400 polymer microspheres (100-300 micron) with varying functionality (-OH, $-NH_2$, -COOH, CH_3) have been shown to induce osteogenesis ($-NH_2$) and chondrogenesis (-OH) in MSCs [133].

Curran *et al.* (2006) showed that, of several functionalities tested (bare glass, -OH, $-NH_2$, -COOH, CH_3, SiH), MSCs were maintained (bare glass, CH_3) or osteogenesis ($-NH_2$, SiH) or chondrogenesis induced (-OH, -COOH). The role of CH_3 functionality was further explored with the added dimension of varying number (1-3 CH_3 groups per silane) and alkyl chain length (8 & 18 carbon). The surfaces influenced fibroblast growth factor release which increased for dichlorodimethylsilane surfaces and resulted in lower expression of MSC markers. Conversely, dithyloctylsilane-modified surfaces increased MSC marker expression [142]. The work further examined the role of topology and functionality, and demonstrated that islands of 65-70 nm Ø with a pitch of 280 nm was optimal for cell attachment [140]. The mechanism for this response was attributed to the requirement of RGD spacing of >70 nm for effective cell attachment and spreading [140]. Such studies demonstrate the importance of the collective local environment, as the medium and surface interact to provide an environment that facilitates a given response. This can be extended to the point of identifying properties with potential application in tissue culture and biomaterial design [91, 133].

4.3 Limitations of Existing Studies: Our understanding of material-induced responses revolves around two themes which relate to two types of surface treatment. Topological studies favor mechanotransduction (often under-emphasizing the chemical nature of the material explored), whereas studies based on chemical modification emphasize differential adsorption of proteins or other biomolecular cues. The mechanistic processes (protein adsorption, focal adhesion, cell tensioning etc.) all appear to form part of the same overarching mechanism - mechanotransduction [143]. Although individual studies justifiably highlight particular elements of a wider system, attempts to unify the separate components of mechanotransduction is perhaps missing from current studies.

Although we have a reasonable understanding of what fundamental topological and chemical features can achieve in culture, we are still some way from a comprehensive understanding of how material properties influence the cell, the mechanisms in action and how material generated environments can be formed. Key questions include how the density of functional groups influences responses and the relative influence of the individual properties of a material presenting several different attributes (e.g. different functionalities and varying topology with functionality) effect a response, though this work has started [128, 129, 140]. Topology and surface chemistry are not universally studied in a well-controlled manner due to material-specific challenges in processing e.g. processing of metals such as the titanium alloys used routinely in the clinic. However, existing studies on these metals do show that variation of material properties influences cell response [144, 145]. Identifying how material cues in 2D platforms translate into 3D platforms is another key area and, ultimately, a predictive understanding of how small changes in surface property such as the orientation of chemical groups etc. influence protein and cell response is required.

5.0 A Perspective on the Future of Tissue Culture Materials:

To remain relevant, tissue culture must continue to improve in order to better represent the cells, organs, biological processes and pathologies that it tries to model. Improvements are being simultaneously derived from three general directions; improved cell lines, improved media and new materials/technologies to support cell expansion/maintenance. However, increasing the cost and complexity of tissue culture acts as a barrier to uptake, and innovative new materials can be proprietary and poorly understood in comparison to existing materials.

Developing materials for biomedical applications which incorporate active biological components is a growing area. While offering great potential, cautionary cases have appeared, in that high doses of powerful biological agents (e.g. bone morphogenetic protein 2), combined with an incomplete understanding of biomolecule-biomaterial affinity have resulted in a negative outcome for some patients such as uncontrolled ectopic calcification [146]. Although many studies use transformative media, materials-based technologies have started to emphasize the advantage that they have in being able to achieve responses without the addition of bioactive molecules [10, 140]. As technologies manipulating cell and tissue development become more widely applied *in vivo*, the risk of uncontrolled proliferative or transformative events becomes a concern. However, the potential of materials to form tailored self-assembling (and ideally self-limiting) microenvironments in concert with the ready supply of biomolecules and tissue already available within the body could have great potential. In order to further this approach, studies to understand long-range effects; e.g. how material based cues can propagate to cells beyond the immediate influence of the surface generated microenvironment are required.

With regard to the area of tailored local microenvironments, these may be particularly useful for better understanding the differences between diseased and normal tissue. A concept that has been given considerable importance in cancer research is the effect of the local microenvironment on the pathogenesis of cancer and associated metastasis [147]. Materials Science, *via* our ability to finely control the surface and its associations with biomolecules such as proteins in the culture environment, offers considerable potential for answering these questions.

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Notes

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ABBREVIATIONS

3Rs, replacement, reduction and refinement principles; ANSC(s), adult neural stem cell(s); BAEC(s), bovine aortic endothelial cell(s), BSA, bovine serum albumin; CAMs, cell adhesion molecules; CHO, Chinese hamster oocytes; ECM, extracellular matrix; Fn, fibronectin; Fb, fibrinogen; hADSC(s), human adipose stem cell(s); hESC(s), human embryonic stem cell(s); MSC(s), mesenchymal stem cell(s); NSC(s), neural stem/progenitor cell(s); PEG, polyethylene glycol; RBM, rat bone marrow; RGD, arginylglycylaspartic acid; SAM, self-assembled monolayer; TCPS, tissue culture polystyrene; WCA, water contact angle.

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The importance and clinical relevance of surfaces in tissue culture

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