Materials for the Stem Cell Factories of the Future

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The materials community is now identifying polymeric substrates that could permit translation of human pluripotent stem cells (hPSCs) from lab-based research to industrial scale biomedicine. Well defined materials are required to allow cell banking and to provide the raw material for reproducible differentiation into lineages for large scale drug screening programs and clinical use, wherein >1 billion cells for each patient are needed to replace losses during heart attack, multiple sclerosis and diabetes. Producing this number of cells for one patient is challenging and a rethink is needed to scalable technology with the potential to meet the needs of millions of patients a year. Here we consider the role of *materials discovery*, an emerging area of materials chemistry that is in a large part driven by the challenges posed by biologists to materials scientists[1-4].

1 **Evolution of stem cell culture**

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3 The term human pluripotent stem cells (hPSCs) describes both human embryonic

4 stem cells (hESCs), typically derived from the inner cell mass of pre-implantation

5 embryos[5], and human induced pluripotent stem cells (hiPSCs) derived by epigenetic

6 reprogramming of somatic cells with stem cell-associated factors[6]. Because hPSCs

7 can self-renew for months, if not years, in culture and can be induced to differentiate

8 into all three germ layers, they provide immense potential for regenerative medicine

9 and drug development, as well as providing new *in vitro* models of genetic disease[7].

10 However, for biomedical applications to be realized, defined culture conditions need

11 to be established to eliminate batch variability and xenogenic contaminants.

12 Furthermore, scalable culture systems are required[8-9]. For adherent culture systems,

13 scalability is often achieved by increasing the surface area of a growth substrate in T-

14 75 flasks (75 cm^2 growth area) without compromising biological performance as

15 measured by pluripotency markers.

16 Compared to a cell derived proteinaceous mixture such as Matrigel[10], polymers are

17 reproducible and amenable to large scale manufacture, e.g. injection molding is

18 currently used to form tens of millions of tissue culture vessels per year. Consequently

19 there are significant efforts to identify synthetic strategies with the ultimate aim being

20 an inexpensive polymer that can be used off the shelf without pre- adsorption of

21 proteins or immobilization of other biomolecules such as peptides. Furthermore, a

22 fully synthetic growth substrate would be amenable to automated robotic cell culture,

23 paving the way for stem cell factories manufacturing billions of hPSCs suitable for

24 clinical use.

1   This review describes the development of growth substrates for hPSC culture from

2   cell extracts to polymeric-derived materials and assesses the cost and scalability

3   issues associated with the most recent advances in hPSC with a particular focus on

4   materials discovery.

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7   **Feeder layers to support hPSC growth**

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9   Over the last 15 years, progress towards more precisely defined culture systems for

10   hPSCs has been made, summarized in Table 1. Initial reports of hPSC culture

11   employed feeder layers of mouse embryonic fibroblasts (MEFs) to support the self-

12   renewal of hPSCs. Feeder layers provide a source of extracellular matrix (ECM)

13   proteins and growth factors such as vitronectin, transforming growth factor β (TGF-β)

14   and Laminin-511 that aid hPSC proliferation and self-renewal. In 1998, Thomson *et*

15   *al.* employed a MEF feeder layer to support hPSC growth in a medium of 80%

16   Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine

17   serum (FBS), 1mM glutamine, 0.1mM β-mercaptoethanol and 1% nonessential amino

18   acids (NEAA). However, the use of non-human (xeno) feeder layers and animal-

19   derived serum such as FBS represent a potential source of pathogens such as

20   endogenous retroviruses and xeno epitopes such as nonhuman sialic acid, N-

21   glycolneuraminic acid (Neu5Gc)[11]. Factors such as Neu5Gc, a monosaccharide, can

22   induce an immune response upon transplantation of hPSCs cultured using xenogenic

23   methods and limit their use to *in vitro* applications.

24

Table 1 | Milestones in hPSC culture and growth substrate materials discovery.

Year	Description
1998	First hESCs harvested from blastocyst and cultured on mouse-derived feeder cells[5]
	Combinatorial polymer library used to screen for structure-property correlations[69]
2001	First report of hESC culture on Matrigel using feeder-free conditions[11]
2004	First report of hESC culture on Fibronectin matrix using feeder-free and serum-free conditions[25]
	First polymer microarray used to screen for hESC growth and differentiation[52]
	Subtle changes in polymer chemistry shown to influence protein adsorption behaviour[62]
2006	First report of defined hESC culture in feeder- and xeno-free conditions[17]
2007	ROCK inhibitor employed to reduce cell apoptosis during cell passaging[26]
	High throughput surface characterization of a polymer microarray[72]
2008	Vitronectin and isoforms of Laminin identified from extracellular matrix to support hESC growth[41]
	Surface wettability of a combinatorial polymer microarray modelled using multivariate analysis[73]
2010	Long-term self-renewal on Laminin-511 surfaces in defined medium[43]
	Synthetic polymer surface employed in defined medium[47]
	Surface chemistry of polymer surfaces shown to influence hESC growth[53]
2011	Long-term hESC self-renewal on polymer surface achieved in defined medium[48]
2012	Human embryoid body cell adhesion to a combinatorial polymer library modelled using molecular descriptors[70]
	Long-term sefl-renewal of hPSCs on Laminin E8 surfaces in xeno-free defined medium[42]
2013	Long-term hESC self-renewal and thermally-triggered passaging achieved using thermoresponsive hydrogel in defined medium[55]

Feeder-free hPSC culture

To produce hPSCs that are safer and more useful in clinical applications, feeder-free culture systems have been developed. The need to replace feeder layers with alternative growth substrates has driven a huge research effort in the discovery of materials that support the long-term self-renewal of hPSCs. Initial work on feeder-free

systems in 2001 employed animal-derived growth substrates such as Matrigel in combination with a MEF-conditioned medium (MEF-CM)[10]. Matrigel is harvested from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells and consists of a complex mixture of various extracellular matrix (ECM) proteins, proteoglycans and growth factors[12]. Unfortunately, culture systems consisting of undefined growth substrates like Matrigel and MEF-CM exhibit batch-to-batch variability. These elements within a culture system make understanding and controlling the cell-material interface difficult, a prerequisite to developing a scalable and reproducible hPSC culture system. Furthermore, batches of Matrigel have been contaminated with lactate dehydrogenase-elevating virus (LDV). This highlights safety concerns with xenogenic media components, although this particular pathogenic risk can be avoided with the use of Geltrex, an undefined LDV-free growth substrate (Table 2)[11, 13-24]. Although, Matrigel can now also be purchased LDV-free.

Concerns over xenobiotic contamination have prompted the development and use of serum-free media in combination with growth substrates containing recombinant proteins[25]. In 2004, Amit *et. al.* successfully demonstrated the self-renewal of I3, I6 and H9 hPSC lines on a fibronectin matrix using a serum replacement consisting of various growth factors known to play a role in maintenance of pluripotency. Basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGFβ1) and leukaemia inhibitory factor (LIF) were tested in different mixtures. A combination of TGFβ1 and bFGF with and without LIF was able to maintain pluripotency and retain normal hPSC features on a human fibronectin growth substrate. However, growth rates and cloning efficiencies of all combinations were inferior to MEF controls.

Table 2 | Commercialized growth substrates and culture media for hPSC culture.

Component	Product	Defined	Synthetic	Xeno-free	Origin	Form
Growth substrate	Matrigel™, BD Biosciences[11]	✗	✗	✗	Cell extract	Gel
	Geltrex™, Invitrogen	✗	✗	✗	Cell extract	Gel
	Synthemax™, Corning[13]	✓	✓	✓	Peptide-polymer conjugate	powder/coated cultureware
	StemAdhere™, Primorigen Biosciences[14]	✓	✓	✓	Recombinant protein (E-Cadherin)	Liquid
	NunclonVita™, Thermo Scientific[15]	✓	✓	✓	Plasma treated polystyrene	Cultureware
	CellStart™, Invitrogen[16]	✓	✓	✓	Humanized protein mixture	Liquid

Component	Product	Defined	Feeder-free	Xeno-free	Serum-free
Culture medium	HEScGRO, Millipore	✓	✗	✓	✓
	mTeSR1™, STEMCELL Technologies[17]	✓	✓	✗	✓
	TeSR™2, STEMCELL Technologies[17]	✓	✓	✓	✓
	StemPro™, Invitrogen[18]	✓	✓	✗	✓
	NutriStem™, STEMGENT[19]	✓	✓	✓	✓
	E8™, GIBCO[20]	✓	✓	✓	✓
	XVIVO™ 10, Lonza[21-22]	✓	✓	✓	✓
	RegES[23]	✓	✗	✓	✓
	hESF9[24]	✓	✓	✓	✓

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3 A major milestone in hPSC culture was the development of a defined culture medium

4 called TeSR1 published in 2006[17]. The essential ingredients within the TeSR1

5 medium were the proteins basic fibroblast growth factor (bFGF) and transforming

6 growth factor beta (TGFβ), Lithium Chloride, γ-aminobutyric acid (GABA) and

7 pipecolic acid. Cell lines H1 and H9 were both shown to self-renew for more than 10

8 passages on a xeno-free growth substrate consisting of human collagen IV,

9 fibronectin, laminin and vitronectin. The derivation of two new hPSC lines, WA15

10 and WA16, was also achieved using TeSR1. However, WA15 became trisomic (three

1　chromosomes as opposed to two) for chromosome 12 between 4 and 7 months in

2　culture. A variant of the TeSR1 culture cocktail has been commercialized as mTeSR1,

3　employing BSA and zebrafish bFGF, for a cheaper alternative to the xeno-free culture

4　medium. To further aid hPSC culture, supplements such as Rho-associated kinase

5　(ROCK) inhibitors have been employed to reduce dissociation-induced cell apoptosis

6　when working in defined medium[26]. However the impact of these inhibitors and their

7　long-term effects on hPSCs are yet to be understood.

8

9　High throughput screening has been used to discover small molecules that improve

10　hPSC survival and self-renewal, to improve hPSC culture methodology. In 2010 Xu *et*

11　*al.* published a high throughput chemical screen of 50,000 synthetic compounds to

12　identify small molecules added to the culture media that promoted hPSC survival after

13　trypsin dissociation from a Matrigel substrate[27]. Thiazovivin (Tzv) and Pyrintegrin

14　(Ptn) were both found to dramatically increase cell survival versus DMSO controls.

15　High throughput screening of small molecules can rapidly identify essential

16　ingredients within current culture media employed in hPSC culture and be used to

17　reduce the number of components within the hPSC culture system (Box 1)[26-40].

18　Furthermore, this screening approach can identify novel culture medium supplements

19　to aid the survival and self-renewal of hPSCs in defined culture conditions.

20

Protein-based growth substrates

Following the identification of mixtures of ECM proteins as adsorbates required for hPSC growth and self-renewal, effort was focused on identifying which proteins are most effective with specific media. Miyazaki *et al.* identified the laminins (LN)-111, -332 and -511 as successful substrates for hPSC culture when used in combination with MEF-CM[41]. The hPSC lines KhES-1 and KhES-3 were found to express pluripotency markers after 10 passages and showed equal growth on LN-332

compared to Matrigel at 72 hours. The utility of these substrates was attributed to their high affinity for the $\alpha_6\beta_1$ integrin expressed on hPSCs. More recently, analogues of these substrates employing laminin E8 fragments (functionally minimal forms of laminin that can bind the $\alpha_6\beta_1$ integrin) have been shown to support hPSC self-renewal in defined xeno-free medium for 10 passages[42]. Laminin-332E8 and -511E8 surfaces were able to support the self-renewal of H9, HES3, KhES-1 (hESC lines) and iPS(IMR90)-1, 253G1 (iPSC lines) in mTeSR1 and StemPro medium. Furthermore, all cell lines displayed normal karyotype at passage 10. These simplified laminin substrates were demonstrated to be successful for hPSC expansion at larger scale, such as T-75 tissue culture polystyrene flasks.

In a similar study, Rodin *et al.* demonstrated the long-term growth of hPSCs on LN-511 coated plates for 20 passages over 4 months in chemically defined O3 medium and xeno-free H3 medium (both variants of TeSR1)[43]. Furthermore, cells were able to attach and migrate over/across the LN-511 coating creating continuous cell monolayers due to their affinity for the $\alpha6\beta1$ integrin. This phenomenon was thought to have aided the long-term self-renewal of the hPSCs. However, passaging the cells required physical removal as clumps from the LN-511 coated plates rather than as single-cell suspensions. This method of passaging is compatible with automation and limits the scalability of this culture system.

Peptide-based growth substrates

Following the use of substrates coated with protein and protein fragments to promote hPSC adhesion, simplified substrates presenting specific peptide sequences have been developed to identify specific interactions at the cell-material interface that mediate

stem cell behaviour. Microarrays of laminin fragments were presented *via* self-assembled monolayers (SAMs) by spotting 18 thiol functionalized peptides onto gold slides to rapidly identify cell-binding potential[44]. H1 and H9 cells that attached and proliferated after 6 days on certain laminin sequences displayed pluripotency markers at similar levels to Matrigel controls. However, scale up from micro array spots and expansion and long-term self-renewal on these surfaces was not demonstrated. Furthermore, the MEF-CM used to culture the hPSC makes this a complex and ill-defined system which will contain many proteins and other biomolecules in the media solution and adsorbed to the surface.

Carboxylate-containing acrylate monomers immobilized on plasma treated polystyrene plates, and subsequently conjugated to various RGD-containing short peptide sequences through the N-terminus, have been used to generate peptide-acrylate surfaces (PAS)[45]. Of the six peptides employed, only two, bone sialoprotein (BSP) and VN-derived peptide, supported hPSC attachment, suggesting that RGD alone is a necessary but not sufficient binding motif. BSP- and VN-PAS were able to demonstrate long-term self-renewal (>10 passages) and were scalable to 75 cm^2 flasks. High surface density of the supportive peptide was required to achieve growth rates similar to Matrigel, with concentrations of BSP ranging from 0.75 - 1mM yielding 6 – 9 pmol/mm^2 peptide density. VN-PAS coated flasks seeded with hPSCs and cultured for 4 days in defined medium showed uniform cell distributions, typical morphology and expressed the pluripotency marker OCT4. This substrate has been developed commercially and marketed as Synthemax (Table 2)[13], however, the biological components used in this substrate means that it is expensive (*ca*. $100/T75 flask) compared to the widely used laboratory growth substrate Matrigel (*ca*. $15/T75

flask). Another example of a growth substrate that has been commercially developed

is StemAdhere[14]. This growth substrate employs a fusion protein of the IgG Fc

domain and E-cadherin, a Ca^{2+} dependent cell-cell adhesion molecule, which is

coated onto polystyrene plates. This substrate was able to support long-term culture of

H9 cells (90 passages) in mTeSR1 medium. Similarly to Synthemax, the recombinant

nature of StemAdhere increases the expense of using these substrates significantly.

The materials cost alone to produce 1 billion hPSCs (an approximation for a single

patient intervention) is estimated to be *ca.* $10,000 and $15,000 for Synthemax and

StemAdhere respectively (Table 3). This cost is likely to be prohibitive for cell

expansion for clinical and biomedical use and is considerably greater than that using

Matrigel, which is estimated to be *ca.* $1,500 (excluding the cost of cells and media)

to produce 1 billion hPSCs. Phase 1 trials have been passed by regulators using

Matrigel as an expansion substrate, but the exact meaning of GMP is still evolving for

hPSC technologies and as more defined systems become available they are likely to

be insisted upon.

Table 3 | Recent developments in defined growth substrates for adherent hPSC culture.

Growth substrate	Culture medium	hPSC line	Passages	Passaging method	Pluripotency criteria	Karyotyping	Cost / Scalability[a]	Cost per 1 billion hPSCs	Substrate characterization
Synthemax[13] (peptide-polymer conjugate)	X-VIVO 10, 80 ng/mL hrbFGF 0.5 ng/mL hrTGF-b	H1 H7	10	collagenase	OCT4 SSEA4 teratomas	data not shown	$380 / 10mg $80 / 6 well plate $100 / T75 $295 / T225 ***	~$10,000[b]	Fluorescence
StemAdhere[14] (Recombinant E-Cadherin)	mTeSR1	H1 H9 iPSC	>60	accutase	OCT3/4 ZFP42 SSEA4 NANOG	H9 normal	$100 / kit $22 / 0.5mg **	~$15,000[c]	Fluoresence
Peptide-SAM[49] (Synthetic peptide)	mTeSR1 + ROCK	H1 H7 H9 H13 H14 IMR-90-1	6 (H1) 6 (H7) 19 (H9) 14 (H13) 17 (H14) 7 (IMR-90-1)	manually	OCT4, SSEA-1 SSEA-3, SSEA-4 SOX2, TRA-1-60 TRA-1-81 teratomas	Trisomy on chromosome 17 (H14), other lines normal	Expensive *	>$15,000[d]	Fluoresence
Polymeric growth substrates									
PMVE-alt-MA[46]	StemPro + StemPro suppl. with DMEM-F12 (2% (v/v) BSA)	HUES1 HUES9 iPSC	5	accutase	OCT4 NANOG teratomas	HUES1 and HUES9 normal	Inexpensive ***	~$1,010[e]	FT-IR
PMEDSAH[47]	mTeSR1, StemPro suppl. with DMEM-F12 + L-glutamine, 15 mM HEPES	BG01 H9	3 (BG01) 10 (H9) (StemPro)	mechanical	OCT3/4 SOX2 + EB (BG01) OCT3/4, SOX2, SSEA4, TRA-1-60 TRA-1-81 teratomas (H9)	BG01 and H9 normal	Inexpensive ***	~$1,010[e]	WCA XPS elastic modulus FT-IR
APMAAm[48]	mTeSR1	H1 H9	>20	collegenase	OCT4, SSEA4 + EB	H1 and H9 normal (p10)	Inexpensive ***	~$1,010[e]	WCA XPS
15A-30%[53]	mTeSR1 (pre-adsorption of HSA to polymer)	BG01 WIBR3	5	collagenase	OCT4 SSEA4 TRA-1-60 NANOG teratomas	BG01 and WIBR3 normal	Inexpensive ***	~$1,010[e]	WCA elastic modulus surface roughness ToF-SIMS
UV/Ozone[54] treated TCPS	mTeSR1 + ROCK (pre-adsorption of hrVN, 20% HSA or 20% FBS to polymer)	BG01 WIBR1 WIBR3	>10	collagenase accutase	OCT4 SSEA4 SOX2 NANOG teratomas	normal >p5 with ROCK (abnormal at p5 without ROCK)	Inexpensive ***	~$1,000[f]	XPS rheology
HG21[55]	mTeSR1	RH1 H9	>20 (RH1) 9 (H9)	thermally induced	OCT3/4 NANOG SOX2 SSEA4 TRA-1-60 EB teratomas	deletions/ duplications on chromosomes 8, 9, 13, 20 (p21)	Inexpensive ***	~$1,010[e]	ToF-SIMS XPS

[a]Scalability determined by the ability to synthesize the material in large quantity for manufacturing scale hPSC production in a cost-effective and timely manner, [b] estimated using 100 x coated T75 flasks required to achieve 1 billion hPSCs, [c] estimated by 150 kits required to coat a sufficient number of 6 well plates to achieve 1 billion hPSCs, [d] estimated to be at least as expensive as a similar recombinant protein-based substrate such as StemAdhere, [e] estimated by the cost of commercial monomers of *ca.* $0.5/g and 50mg of dissolved polymer required to coat 350 T75 flasks, [f] estimated by the cost of 350 T75 flasks.

Polymer-based growth substrates

Systems that we classify as *scalable* are those which can be used to produce billions of cells in an economical and safe manner for many patients. For example, the material cost of producing 1 billion hPSCs using a Matrigel coated substrate is *ca.*

1    $1,500, far cheaper than Synthemax and StemAdhere (Table 2). A benchmark in the

2   pharmaceutical industry to screen differentiated stem cells for drugs is to achieve a

3   cost of <$1 per well (in a 96 well plate). This is not currently achievable for

4   pluripotent stem cell culture. The peptide-polymer derived substrates mentioned

5   previously are amenable to commercial development but ideally the substrate should

6   be fully synthetic using readily synthesized and cheap monomers.

7   Synthetic substrates that are polymer-based are generally inexpensive to manufacture

8   and have shown broad utility commercially. Brafman *et. al.* employed a polymer

9   microarray consisting of 91 commercially available and pre-synthesized polymers to

10   screen for hPSC attachment in StemPro medium[46]. A broad range of polymer

11   backbones and side chain functionalities were screened, including styrenes, acrylates

12   and acrylamides spotted onto acrylamide coated glass slides. Of the initial hits

13   identified by high OCT4 and NANOG expression using fluorescence microscopy to

14   identify pluripotency, one polymer, poly(methyl vinyl ether-alt-maleic anhydride)

15   (Figure 1d(ii)), was able to support hPSC attachment and self-renewal for >5

16   passages using StemPro medium. Scalable expansion of hPSCs on this polymeric

17   substrate was not demonstrated beyond cell expansion on polymer-coated slides.

18

19   Six unique acrylate-based surfaces have also been prepared via an ozone-activation of

20   tissue culture polystyrene (TCPS) and subsequent surface-initiated polymerization

21   with a range of acrylate monomers[47]. One of these materials, poly [2-

22   (methacryloyloxy)ethyl   dimethyl-(3-sulfopropyl)   ammonium   hydroxide]

23   (PMEDSAH), was able to support the long-term culture of hPSC in serum-free

24   defined mTeSR1 medium (including protein supplement) (Figure 1d(iii)). H9 cells

25   were supported through 10 passages using StemPro medium, showed normal

1 karyotype and expressed similar levels of pluripotency markers to cells cultured on

2 Matrigel. However, no scalability was demonstrated using this substrate material.

3 Irwin *et. al.* developed a methacrylamide based coating (Figure 1d(iv)) which was

4 grafted to TCPS dishes using a photoinitiated addition polymerization[48]. H1 and H9-

5 hOCT-pGZ hPSC lines were cultured in mTeSR1 medium for 10 passages,

6 maintained typical cell morphology and grew in colonies similar to Matrigel cultured

7 cells. Bovine serum albumin (BSA) was proposed to play a crucial role in hPSC

8 attachment achieved in the culture medium. Quartz crystal microbalance with

9 dissipation (QCM-D) experiments were used to identify BSA adsorption to the

10 growth substrate from the mTeSR1 medium.

11
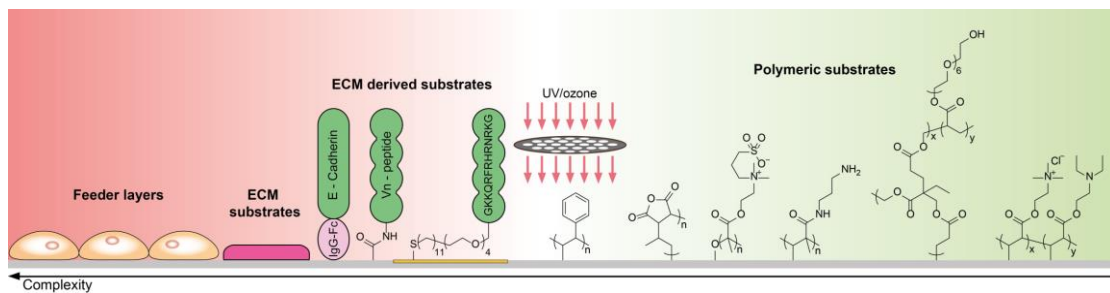
13 **Figure 1 | The development of hPSC growth substrates.** (a) Feeder layers of MEFs

14 to support cell adhesion and to condition the culture medium with ECM proteins to

15 facilitate hPSC self-renewal. (b) Surface coating with an undefined ECM protein-

16 containing mixture, e.g. Matrigel. (c) Functional epitopes of ECM components

17 immobilized to the surface to encourage hPSC attachment and self-renewal. (d)

18 Polymeric growth substrates to provide an environment to adsorb essential ECM

19 proteins from culture medium.

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High throughput materials discovery for stem cell culture

The defined growth substrates for adherent hPSC culture surveyed above have limitations, and consequently the search for new materials for hPSC culture continues. As we lack mechanistic understanding why cells respond to materials and media, high throughput methodologies have been employed to screen as wide a combinatorial chemical space as possible for materials supporting pluripotent cell number expansion.

Surface modification strategies such as self-assembly have been used to present molecules capable of binding to cell surface integrins with high spatial resolution (Derda *et al.*[44]). Arrays of peptide-substituted alkanethiols have been prepared as self-assembled monolayers on gold surfaces[49]. The molecules screened included peptides containing RGD and glycosaminoglycan binding epitopes, the most successful of which being a heparin-binding peptide derived from vitronectin (VN) (GKKQRFRHRNRKG). This peptide was able to support long-term self-renewal of hPSCs at peptide densities of 0.5-25% (% peptide-substituted alkane thiol in mixed SAM monolayer) when combined with ROCK inhibitor or cyclic RGD peptide. This peptide was used to functionalize glass and gold coated slides. Furthermore, biotinylated-GKKQRFRHRNRKG was used to functionalize steptavidin-coated TCPS dishes in a facile manner to reduce the cost of employing this peptide. However, hPSC expansion was not demonstrated over large areas, e.g. a culture flask. Pre-synthesized polymer libraries were printed as microarrays by Anderson *et al.*[50] employing the concept of combinatorial polymer libraries pioneered by Kohn et al in 1997[51]. For high throughput materials discovery, this has the limitation that polymer synthesis is time consuming, reducing the diversity of such arrays and slowing the

1   follow up of leads generated on subsequent arrays. Ideally, the evolution from the

2   initial screen to future generation arrays arises from hypotheses being formed from a

3   first generation array that are subsequently tested in generations that evolve rapidly

4   according to the results generated. On-slide synthesis of polymer microarrays was

5   achieved by Anderson *et al.* in 2004[52], allowing rapid synthesis of acrylate polymers

6   by combinatorial mixing of liquid monomers printed onto a hydrogel coated slide

7   prior to UV photoinitiated free radical polymerization. A strength of polymer

8   microarrays is the ability to rapidly assess cell response to a large polymer library,

9   which when coupled with the ability to perform surface analysis of the library on the

10   array, allows cell-material surface interactions to be investigated rapidly[3].

11

12   Subsequently, several groups have used polymer microarrays to screen surface

13   chemistries for hPSC attachment in a variety of culture media[46,52,54]. Mei *et al.*

14   screened a number of generations of polymer microarray, starting with a library of

15   496 unique materials formed by mixing 16 acrylate 'major' monomers with 6 'minor'

16   monomers that were contact printed onto a poly(2-hydroxyethyl methacrylate)

17   (pHEMA) coated substrate and polymerized *in-situ* using UV irradiation[53]. Polymers

18   with potential as supports for pluripotent stem cells were identified by their ability to

19   support the clonal growth of BG01-Oct4-GFP$^+$ cells from very low initial seeding

20   densities over 7 days in MEF-CM (arrays pre-conditioned with FBS). High content

21   fluorescent microscopy was used to quantify cell response to the individual polymer

22   spots using OCT4 GFP. High throughput surface characterization of the microarray

23   was used to quantify the materials chemistry and properties such as wettability and

24   indentation elastic modulus, which were subsequently compared to cell performance

25   to identify the controlling surface factors. Colony-formation efficiency, a measure of

the number of colonies formed at day 7 from the initial low cell attachment on day 1,

was used to quantify the performance of the hit substrates pre-adsorbed with ECM

proteins. Human vitronectin pre-adsorbed surfaces gave the highest colony formation

efficiency in mTESR1 medium maintaining pluripotency for 10 passages over 8

weeks on microarrays comprising the hit materials (monomer 9 and 15A), however,

only medium-term passaging (>5 passages over 1 month) was reported on surfaces

pre-incubated with human serum albumin (HSA) and no scalability beyond

microarray spots was demonstrated for the hit materials (Figure 1d (v)).

A subsequent study used UV-ozone modification of polystyrene which maintained

pluripotency for >10 passages on surfaces conditioned with HSA or human Vn in

mTESR1 medium[54]. This growth substrate represents an attractive, cost-effective and

simple route amenable to scale up, although Vn is still required as a pre-adsorption

step for both approaches, which increases the cost of employing this culture system

(Figure 1d (i)).

More recently, Zhang *et al.* produced a microarray of 609 different thermoresponsive

polymers by ink-jet printing 18 acrylate and acrylamide monomers with a crosslinker

in various ratios[55]. The best performing material, an acrylate copolymer consisting of

trimethylammonium chloride and diethylamino side-groups (HG21), supported the

long-term self-renewal of RH1 cells (hPSC line) in mTeSR1 medium (>20 passages).

Karyotype analysis of the RH1 cells at passage 21 found chromosomal abnormalities.

This highlights the need for characterization of hPSCs at high passage number as

abnormalities can occur after several passages which render the hPSCs unusable for

clinical application.

The thermoresponsive nature of the copolymer hydrogel permitted cellular

detachment by reducing the culture medium temperature to 15°C for 30 minutes. This

1 step may be useful in automated stem cell expansion systems. Growth rates of RH1

2 cells on hydrogel-coated coverslips were significantly lower than Matrigel controls;

3 RH1 cells took 8-10 days to reach 80% confluence on the hydrogel coating as

4 opposed 4-5 days on Matrigel in mTESR1 medium. Xeno-free culture medium was

5 not used and scalability of this growth substrate was not demonstrated beyond coating

6 of cover slips. The thermo switch of the gels was characterized by bulk rheology

7 measurements of materials on cover slips.

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10 **Mechanism of cell response to surface cues**

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12 Many research groups have directed their efforts towards understanding the effect of

13 materials on the regulation of stem cell behavior by designing substrates with

14 particular chemistries, compliances, topographies, or containing biologically relevant

15 moieties[49,56-57]. Cell adhesion molecules that govern cell-matrix and cell-cell

16 interactions play a crucial role in the long-term maintenance and self-renewal of

17 adhered hPSCs. The identification of cell surface integrins that can engage with

18 Matrigel (β_1) and Vitronectin ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) -coated substrates has enabled an

19 understanding of how hPSC pluripotency is maintained after they have adhered to

20 substrates[42,53,58-59]. Cell-cell interactions mediated by cadherins and their role towards

21 hPSC behavior has been extensively studied over recent years; see for example Li *et*

22 *al.* for a recent review of this area[60]. Specifically, substrates presenting E-cadherin

23 have been commercialized as StemAdhere and have proven to be useful substrates for

24 hPSC expansion[14].

1 Adsorption of individual proteins on polymeric substrates and the subsequent effect

2 upon cellular performance has been well studied; see for example Koenig *et al.*[61] and

3 Weber *et al.*[62]. However, understanding which proteins adsorb from complex protein

4 containing media onto materials, and the conformation they adopt on adsorption to a

5 synthetic surface is still not yet possible, yet it is an essential element in gaining an

6 understanding of material performance in protein containing culture conditions. Such

7 knowledge will ultimately direct materials discovery towards improved substrates for

8 hPSC culture.

9 The topography and elastic modulus of synthetic substrates has been shown to

10 influence the differentiation of stem cells[63-65]. These studies highlight the importance

11 of physical as well as chemical properties in regulating self-renewal and

12 differentiation in future culture systems used for large scale manufacture of hPSCs.

13 Recently, the heparin-binding peptide GKKQRFRHRNRKG (see earlier discussion of

14 this peptide) was attached to hydrogel-based surfaces of various moduli to enable

15 mechanical control of hPSC self-renewal[67]. Immobilization of the peptide on the

16 hydrogel substrates was achieved by a chemoselective reaction between maleimide

17 moieties and the peptide terminated with a cysteine residue. Only stiff hydrogels (10

18 kPa) were able to maintain hPSC pluripotency, which was facilitated by the activation

19 of the Yes-Associated Protein (YAP) and transcriptional coactivator with PDZ-

20 binding motif (TAZ). The YAP/TAZ are implicated in hPSC pluripotency and the

21 hydrogel exhibiting a Youngs Modulus of 10 kPa, determined by force indentation

22 measurements using AFM, was most effective at inducing YAP/TAZ nuclear

23 localization.

24

25

1   **Modelling and Predicting Material Performance**

2

3   To support experimental materials discovery, computational methods of predicting the

4   role of materials in encouraging cellular attachment have been explored[68] (Figure 2).

5   An early example of this was demonstrated for fibroblasts by Brocchini *et al.* who

6   identified polymer-cell response relationships within a combinatorial library of pre-

7   synthesized copolymers spotted to form a micro array[69]. Linear correlations were

8   observed between fibroblast proliferation and polymer surface hydrophobicity for a

9   subset of the library of polymers. More recently human embryoid body cell adhesion

10   to a library of polymers has been linked to molecular descriptors indicating that

11   computational approached may be used to guide the design of materials production

12   for experimentation with stem cells (Figure 2).[70]
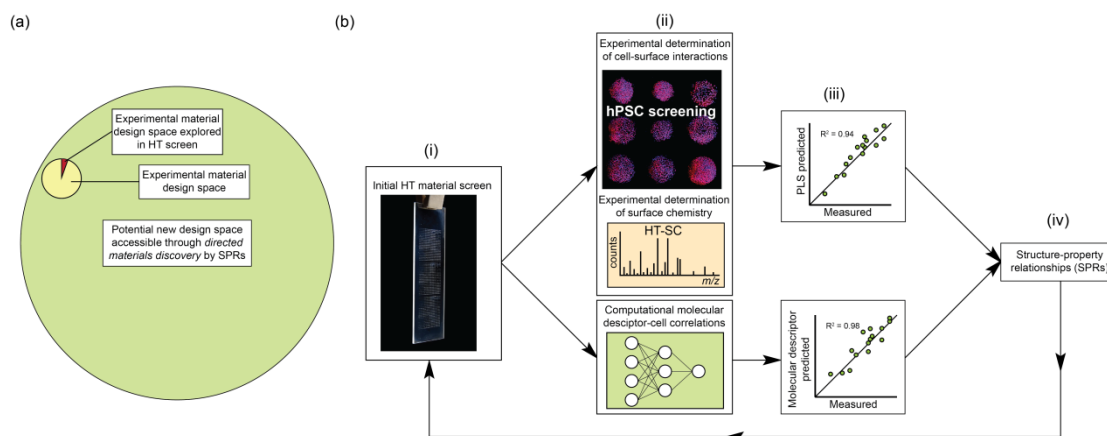
13

14   **Figure 2 | High throughput materials discovery.** (a) **Concept**:   The small

15   experimental material design space that can readily be accessed limits the discovery

16   of new materials for hPSC culture. Directed materials discovery can be achieved

17   through advanced modelling methods that enable structure-property relationships

18   (SPRs) to be developed. This has the potential to allow access to materials design

19   space yet to be explored experimentally. (b) **Workflow** (i) High throughput sample

20   formats such as polymer microarrays can be screened for new growth substrates for

1 hPSC culture to access a small proportion of the potential chemical space. (ii) High

2 throughput surface characterization can be employed in parallel to the biological

3 assay. Correlation of the surface analytical data with cell performance using

4 multivariate partial least-squares (PLS) regression links structure to function. Neural

5 networks can identify molecular descriptors correlating with cell response. (iii) The

6 predictive models have the potential to identify material chemistries that could not

7 have been predicted from the experimental data alone. (iv) These advanced modelling

8 techniques can be used to develop SPRs that can be used to explore new materials

9 design space. This process can be performed in an iterative manner until an optimized

10 material has been found.

11

12 Surface chemical measurements can be used to determine the surface chemistries

13 controlling cell attachment to materials with the aid of statistical and machine learning

14 methods employed to aid interpretation of the large data sets. For example, Chilkoti

15 *et al.* characterized 15 oxygen-containing plasma-deposited films using static

16 secondary ion mass spectrometry (SIMS)[71]. A correlation between the positive and

17 negative ion spectra from the materials and endothelial cell growth was determined

18 using multivariate partial least-squares (PLS) regression. The PLS model identified

19 ions within the SIMS spectra that contributed towards high and low cell attachment.

20 Using a related approach, Urquhart *et al*. employed a combination of high throughput

21 surface characterization (surface mass spectral data) and multivariate analysis (MVA)

22 to predict the wettability of 576 polymers in a combinatorial microarray library[72-73]. A

23 statistically valid PLS model between water contact angle (WCA) measurements and

24 spectra obtained using time-of-flight secondary ion mass spectrometry (ToF-SIMS)

25 was obtained. This study demonstrated the utility of MVA techniques such as PLS to

model datasets containing a large number of variables such as ToF-SIMS spectra (each with hundreds of secondary ions) of a polymer microarray (containing hundreds of materials). Following this, Mei *et al.* employed PLS regression to explore the relationship between surface chemistry of a combinatorial polymer microarray and the colony-formation frequency of hPSCs[53]. Good agreement between the measured colony-formation frequency and that predicted from the ToF-SIMS spectra from the material surfaces was found, highlighting the importance of surface chemistry of a material. The approach also helped identify the controlling surface functionalities, to allow improvements from one generation to the next, to obtain the best performing materials from the library of monomers available. The lead polymers were pre-adsorbed with vitronectin before cell seeding. To investigate the role of this step, ToF-SIMS of these protein conditioned polymer surfaces was used to analyze the chemistry of this surface. Strong correlations between cell attachment and protein fragment secondary ions were identified, indicating the synergy existing between the material surface chemistry and the identity and amount of the adsorbed proteins to enable colony-formation. It is known from blocking experiments that the role of protein adsorption to polymeric growth substrates is pivotal to facilitate hPSC attachment and self-renewal *via* engagement with cell surface integrins[53]. Understanding which proteins adsorb from complex protein containing media to materials and their conformation is an essential component in interpreting material performance in protein containing culture conditions and will ultimately lead materials discovery towards better substrates for hPSC culture (Box 2). Although, at this time, unequivocal identification of protein identity and conformation from complex protein containing media is not possible.

Box 2│Challenges in hPSC culture for material scientists.

The move towards defined synthetic growth substrates for hPSC culture has been approached in a concerted manner and primarily driven by biologists aiming to achieve greater reproducibility during cell culture and remove xenogenic components. Materials scientists play an important role in meeting challenges posed by biologists *via* the design of growth substrates capable of achieving high expansion rates of hPSCs whilst maintaining full pluripotency potential and a normal karyotype.

A major challenge in material design is the development of a fully synthetic growth substrate that can adsorb essential proteins in the desired conformation from the culture medium to facilitate hPSC adhesion and expansion. The mechanism of hPSC adhesion to biologically inspired growth substrates that display surface moieties such as RGD-containing peptides is well understood. However, there is gap in knowledge of how biomolecules adsorb to synthetic substrates which is limiting the rational design of improved synthetic growth substrates.

One materials design approach towards functional synthetic growth substrates is to develop synthetic mimics of biological motifs known to be beneficial for hPSC adhesion eg. employing sulfonated synthetic polymers to mimic the functional characteristics of heparin[47]. This hypothesis-driven approach has led to the development of polymer-based growth substrates that can achieve hPSC expansion in defined media[47-48, 53,55].

Another design route for synthetic growth substrates is a combination of high-throughput material and computational screening. It is often not appreciated how vast the possible 'space' of materials that could be synthesized is, and that it is not possible to explore even a tiny fraction of this by experiments. The vast design space of synthetic biomaterials presents many opportunities to discover better synthetic growth substrates. To meet this challenge and explore the chemical combinatorial design space more effectively; computational methods will be needed to complement experimentally derived hypotheses and better inform materials discovery screening. Computational modelling can contribute in several ways. Use of design of experiments (DoE) methods allows the number of experiments that are required to cover a given design space to be minimized. Computational models of the data from these experiments allow the properties of all materials within the design space to be predicted.

If large-scale high throughput methods can be developed that capture sufficient molecular diversity on the materials, these models are capable of wide extrapolation into materials space. Finally, evolutionary methods are beginning to be applied to materials design and discovery. They allow initial sets of promising materials to be evolved towards a desired materials property 'fitness function' in an experimentally efficient way. These methods are among the most efficient at exploring extremely large design spaces. (**Figure 2**).

1
2

Outlook

Human pluripotent stem cells (hPSCs) have present possibilities in a wide variety of applications such as regenerative medicine and pharmaceutical drug screening. In the future, stem cell factories will be required to produce the large numbers of hPSC that will be required to meet the demand for billions of cells typical of regenerative medicine interventions currently in clinical trials. The long-term expansion and self-renewal of hPSCs in xeno- and defined conditions is a prerequisite to achieving this. Xeno- and feeder-free E8 medium has now been commercialized[21] and E6, a medium used to reprogram somatic cells to hiPSCs prior to expansion in E8, is currently under development. For adherent hPSC culture this will also need to be supported by chemically defined substrates that offer high growth rates in a reproducible manner. Suspension hPSC culture has been shown as a promising alternative to adherent hPSC culture. However, refinement of the culture media components and improved growth rates versus adherent systems will need to be demonstrated before suspension culture can be considered as a viable alternative.

The most recently developed defined substrates are polymers and peptides that are applicable to a xeno- and serum-free environment but few are able to support the expansion of hPSCs at levels similar to that of the current gold standard (but undefined) growth substrates, such as Matrigel. A number of novel polymers show promise, but still largely require protein pre-adsorption or display significant limitations in industrial scale up-mainly cost.

1 The search for new materials for adherent hPSC culture has been greatly accelerated

2 by the recent application of high throughput sample screening strategies such as

3 polymer microarrays. Surface characterization and correlative and predictive models

4 make this a powerful approach with which to search for new materials. Development

5 of quantitative structure-property relationships (SPRs) using the results from large

6 experimental libraries linking polymer structure to hPSC performance on materials

7 are likely to broaden the chemical combinatorial space beyond what is currently

8 explored to facilitate the search for better materials for hPSC culture. The materials

9 discovery process can be further aided through combination with high throughput

10 screening of synthetic soluble factors that can replace biologically derived ingredients

11 within hPSC culture media and increase the scalability of such culture systems.

12

13 High throughput materials screening is moving towards the ability to carry out

14 *directed high throughput materials discovery* to allow exploration beyond the existing

15 experimentally investigated chemical space utilizing experimentally determined

16 surface structure-property relationships (sSPRs) and computationally determined

17 molecular descriptors. This will represent a step change in materials discovery

18 capabilities to enable these discovery methods to evolve beyond the constraints of the

19 current experimentally accessible chemical space. Recently, molecular descriptors

20 have been used to predict the response of stem cell attachment and generate sSPRs in

21 silicio, without the need for experimentally determined polymer characteristics[70].

22 Although the "reverse SPR" problem (backing out a polymer structure from an SPR

23 model) has been challenging in the past, exciting new developments in mathematics

24 have recently provided practical methods for designing polymers with optimal

25 properties from structure-property relationships and molecular descriptors[74]. Success

in this endeavor would open up the full range of materials to computational examination to direct synthesis efforts to potentially fruitful areas for experimental exploration. We anticipate that these and the other material discovery approaches covered in this review will provide the new materials necessary for the stem cell factories of the future required to supply emerging stem cell therapies.

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