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Materials for stem cell factories of the future

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Polymeric substrates are being identified that could permit translation of human pluripotent stem cells from laboratory-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. Yet more than 1 billion cells for each patient are needed to replace losses during heart attack, multiple sclerosis and diabetes. Producing this number of cells is challenging, and a rethink of the current predominant cell-derived substrates is needed to provide technology that can be scaled to meet the needs of millions of patients a year. In this Review, we consider the role of materials discovery, an emerging area of materials chemistry that is in large part driven by the challenges posed by biologists to materials scientists.

The term human pluripotent stem cells (hPSCs) describes both human embryonic stem cells (hESCs), typically derived from the inner cell mass of pre-implantation embryos[1], and human induced pluripotent stem cells (hiPSCs), derived by epigenetic reprogramming of somatic cells with stem-cell-associated factors[2]. Because hPSCs can self-renew in culture for months if not years, and can be induced to differentiate into all three germ layers, they provide immense potential for regenerative medicine and drug development, as well as for new *in vitro* models of genetic disease[3]. For biomedical applications to be realized, however, defined culture conditions need to be established in order to eliminate batch variability and xenogenic contaminants. Furthermore, scalable culture systems are required[4,5]. For adherent culture systems, scalability is often achieved by increasing the surface area of a candidate growth substrate in T75 flasks (flasks with 75 cm^2 growth area) without compromising biological performance as measured by pluripotency markers.

In comparison to a cell-derived proteinaceous mixture such as Matrigel[6], polymers are not prone to batch-to-batch biological variations and they are readily amenable to large-scale manufacture; for example, injection moulding is used at present to form tens of millions of tissue-culture vessels per year. Consequently, there are significant efforts to identify synthetic substrates on which pluripotent stem cell expansion can be supported. The ultimate aim would be to have an inexpensive polymer that can be used off-the-shelf without pre-adsorption of proteins or immobilization of other biomolecules such as peptides. Ideally, a fully synthetic growth substrate would be amenable to automated robotic cell culture, paving the way for stem cell factories that could manufacture billions of hPSCs suitable for clinical use.

This Review describes the development of growth substrates for hPSC culture, from cell extracts to polymeric materials, and assesses the cost and scalability issues associated with the most recent advances in hPSC culture, with a particular focus on materials discovery.

Feeder layers to support hPSC growth

Table 1 summarizes progress made over the past 15 years towards more precisely defined culture systems for hPSCs. Initial reports of hPSC culture used feeder layers of mouse embryonic fibroblasts (MEFs) to support the self-renewal of hPSCs. Feeder layers provide a source of extracellular matrix (ECM) proteins and growth factors — such as vitronectin (VN), transforming growth factor β (TGF-β) and laminin-511 — that aid hPSC proliferation and self-renewal. In 1998, a MEF feeder layer was used to support hPSC growth in a medium of 80% Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal bovine serum (FBS), 1 mM glutamine, 0.1 mM β-mercaptoethanol and 1% non-essential amino acids[1]. But the use of non-human (xeno) feeder layers and animal-derived serum such as FBS represents a potential source of pathogens, such as endogenous retroviruses and xenoepitopes (for example, non-human sialic acid and *N*-glycolneuraminic acid (Neu5Gc), a monosaccharide)[7]. Factors such as Neu5Gc can induce an immune response on transplantation of hPSCs cultured using xenogenic methods, and limit their use to *in vitro* applications.

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 biological substrates that support the long-term self-renewal of hPSCs. Initial work on feeder-free systems in 2001 used animal-derived growth substrates (such as Matrigel) in combination with a MEF-conditioned medium (MEF-CM)[6]. Matrigel is harvested from Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, and consists of a complex mixture of various ECM proteins, proteoglycans and growth factors[8]. Matrigel is currently very widely used, but is unfortunately prone to batch-to-batch variability in the same way as all biologically based culture systems such as MEF-CM. This complexity within a culture system makes it more difficult to understand and control the cell/material interface, which are prerequisites 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)[7,9–20]. Subsequently, Matrigel was offered as an LDV-free product.

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

Year	Description
1998	First hESCs harvested from blastocyst and cultured on mouse-derived feeder cells[1].
	Combinatorial polymer library used to screen for structure–property relationships[65].
2001	First report of hESC culture on Matrigel using feeder-free conditions[7].
2004	First report of hESC culture on a fibronectin matrix using feeder-free and serum-free conditions[21].
	First polymer microarray used to screen for hESC growth and differentiation[48].
	Subtle changes in polymer chemistry shown to influence protein-adsorption behaviour[58].
2006	First report of defined hESC culture in feeder- and xeno-free conditions using TeSR1 medium[13].
2007	Rho-associated kinase (ROCK) inhibitor used to reduce cell apoptosis during cell passaging[22].
	High-throughput surface characterization of a polymer microarray[68].
2008	Vitronectin and isoforms of laminin identified from extracellular matrix to support hESC growth[37].
	Surface wettability of a combinatorial polymer microarray modelled using multivariate analysis[69].
2010	Long-term self-renewal on laminin-511 surfaces in defined O3 medium and xeno-free H3 medium[39].
	Synthetic polymer surface used in StemPro medium[42].
	Surface chemistry of polymer surfaces shown to influence hESC growth[49].
2011	Long-term hESC self-renewal on polymer surface achieved in mTeSR1 medium[43].
2012	Human embryoid body cell adhesion to a combinatorial polymer library modelled using molecular descriptors[66].
	Long-term self-renewal of hPSCs on laminin E8 surfaces in TeSR2 medium[38].
2013	Long-term hESC self-renewal and thermally triggered passaging achieved using thermoresponsive hydrogel in mTeSR1 medium[51].

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

Growth substrate

Product	Defined	Synthetic	Xeno-free	Origin	Supplied form
Matrigel, BD Biosciences[7]				Cell extract	Gel
Geltrex, Invitrogen				Cell extract	Gel
Synthemax, Corning[9]	✓	✓	✓	Peptide–polymer conjugate	Powder/coated cultureware
StemAdhere, Primorigen Biosciences[10]	✓	✓	✓	Recombinant protein (E-cadherin)	Liquid
NunclonVita, Liquid Thermo Scientific[11]	✓	✓	✓	Plasma-treated polystyrene	Cultureware
CellStart, Invitrogen[12]	✓	✓	✓	Humanized protein mixture	Liquid

Culture medium

	Defined	Feeder-free	Xeno-free	Serum-free
HEScGRO, Millipore	✓		✓	✓
mTeSR1, STEMCELL Technologies[13]	✓	✓		✓
TeSR2, STEMCELL Technologies[13]	✓	✓	✓	✓
StemPro, Invitrogen[14]	✓	✓		✓
NutriStem, STEMGENT[15]	✓	✓	✓	✓
E8™, GIBCO[16]	✓	✓	✓	✓
XVIVO 10, Lonza[17,18]	✓	✓	✓	✓
RegES[19]	✓		✓	✓
hESF9[20]	✓	✓	✓	✓

Concerns over xenobiotic contamination have prompted the development and use of serum-free media in combination with growth substrates containing recombinant proteins[21]. In 2004, 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 was demonstrated[21]. Basic fibroblast growth factor (bFGF), TGF-β1 and leukaemia inhibitory factor (LIF) were tested in different mixtures. A combination of TGF-β1 and bFGF with and without LIF was able to maintain pluripotency and retain normal hPSC features on a human fibronectin growth substrate. Growth rates and cloning efficiencies of all combinations were, however, inferior to MEF controls.

A milestone in hPSC culture was the development of a defined culture medium called TeSR1, published in 2006[13]. The essential ingredients in the TeSR1 medium were the proteins bFGF and TGF-β, lithium chloride, γ-aminobutyric acid and pipecolic acid. Cell lines H1 and H9 were both shown to self-renew for more than 10 passages on a xeno-free growth substrate consisting of human collagen IV, fibronectin, laminin and VN. The derivation of two new hPSC lines, WA15 and WA16, was also achieved using

TeSR1. However, between 4 and 7 months in culture, WA15 became trisomic for chromosome 12 (three chromosomes as opposed to two). A variant of the TeSR1 culture cocktail has been commercialized as mTeSR1, using bovine serum albumin and zebrafish bFGF, as a cheaper alternative to the xeno-free culture medium. To aid hPSC culture further, supplements such as Rho-associated kinase (ROCK) inhibitors have been used to reduce dissociation-induced cell apoptosis when working in defined medium[22]. The impact of these inhibitors and their long-term effects on hPSCs are yet to be understood.

To improve hPSC culture methodology, high-throughput screening has been used to discover small molecules that improve hPSC survival and self-renewal. In 2010, a high-throughput chemical screen of 50,000 synthetic compounds identified small molecules that, when added to the culture media, promoted hPSC survival after trypsin dissociation from a Matrigel substrate[23]. Thiazovivin and Pyrintegrin were both found to increase cell survival markedly when compared with dimethyl sulphoxide (DMSO) controls. High-throughput screening of small molecules can rapidly identify essential ingredients within current culture media used in hPSC culture, and can be used to reduce the number of components within the hPSC culture system (Box 1; refs 22–36). Furthermore, this screening approach can identify new culture medium supplements to aid the survival and self-renewal of hPSCs in defined culture conditions.

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. The laminins (LN)-111, LN-332 and LN-511 were identified as successful substrates for hPSC culture when used in combination with MEF-CM[37]. 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[38]. Laminin-332E8 and laminin-511E8 surfaces were able to support the self-renewal of H9, HES3 and KhES-1 (hESC lines) as well as IMR90-1 and 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 in T75 tissue-culture polystyrene flasks.

A similar study 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)[39]. Furthermore, cells were able to attach and migrate over/across the LN-511 coating, creating continuous cell monolayers because of their affinity for the $\alpha_6\beta_1$ integrin. This phenomenon was thought to have aided the long-term self-renewal of the hPSCs. But passaging the cells required physical removal of the cells from the LN-511-coated plates, resulting in cell clumps rather than single-cell suspensions. Moreover, this method of passaging is incompatible with automation, limiting 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, substrates presenting specific peptide sequences have been developed to identify and utilize specific interactions at the cell/material interface that mediate stem cell behaviour. To rapidly identify cell-binding potential, microarrays of laminin fragments were prepared via self-assembled monolayers (SAMs) by spotting 18 thiol functionalized peptides onto gold slides[40]. 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

Box 1 | Challenges in hPSC culture for stem cell biologists.

Improving current culture systems for the expansion of hPSCs is essential before the full potential of hPSCs can be realized in clinical applications. To be routinely used, hPSCs would ideally be produced in a good manufacturing practice (GMP)-grade culture system. At present, the definition of GMP in terms of hPSC technologies is still being established, as undefined culture systems have entered phase 1 clinical trials. With the advent of defined substrates, however, regulators will inevitably require all the components of a GMP-grade culture system to be xeno-free, fully defined, and amenable to large-scale production, ideally in an automated process. This presents a challenge in hPSC culture for stem cell biologists. GMP-grade hPSCs would have to be fully characterized following large-scale production to ensure that normal karyotype, proliferation rate, pluripotency-associated-marker profile and differentiation potential had been maintained following long-term culture within the system. The success of hPSC culture systems to produce such cells depends on controlling the dynamic interactions that occur between the hPSCs, the medium components and the growth substrate. As such, there has been much recent investigation into the development of defined synthetic substrates, as well as defined synthetic medium supplements that improve hPSC culture systems.

Traditional medium supplements to aid hPSC culture most commonly consist of biological molecules such as growth factors and serum proteins. Recently, small-molecule chemistries have been shown to influence hPSC behaviour in culture, including differentiation activity and reprogramming potential. Importantly, there have been numerous molecules reported to promote the survival and self-renewal of hPSCs when added to the culture medium (see list below).

These molecules can manipulate hPSC activities by the activation or inhibition of key molecules within signalling pathways including MEK (PD0325901 and PD98059)[24,25], ROCK (Y27632, Thiazovivin, HA-1077 and Pinacidil)[22,23,26,27], FGF (SU5402 and PD173074)[28], ERK (SC1)[29] and GSK3 (Bio, CHIR99021 and Bisindolylmaleimide 1i)[24,30,31]. Small-molecule chemistries thus have great potential as medium supplements in the development of fully defined and cost-effective hPSC culture conditions that are amenable to GMP scale-up.

Small-molecule supplements for hPSC survival and self-renewal

BIO (6-bromoindirubin-3-oxime)[30]
Bisindolylmaleimide 1i[31]
CHIR99021 (Calbiochem)[24]
Geldanamycin[32]
HA-1077 (Fasudil)[26]
ID-8[33]
PD0325901[24]
PD173074[28]
PD98059[25]
Pinacidil[27]
Pyrintegrin[23]
Retinol[34]
SC1 (Pluripotin)[29]
SU5402[28]
SU6656[36]
Thiazovivin[23]
U0126[35]
ROCK Y-27632[22]

microarray spots, and expansion and long-term self-renewal on these surfaces, was not demonstrated. Furthermore, the MEF-CM used to culture the hPSCs makes this a complex, xenogenic and ill-defined system, as it contains many proteins and other biomolecules in the medium that adsorb 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)[41]. Of the six peptides used, only two (bone sialoprotein (BSP) and VN-derived peptide) supported hPSC attachment, suggesting that RGD alone is not a sufficient binding motif. BSP- and VN-PAS were able to demonstrate long-term self-renewal (more than 10 passages), and were scalable to 75-cm^2 flasks. High surface density of

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 10^9 hPSCs	Substrate characterization
Biological substrates									
Synthemax[9] (peptide–polymer conjugate)	X-VIVO 10, 80 ng ml^{-1} hrbFGF, 0.5 ng ml^{-1} hrTGF-β	H1, H7	10	Collagenase	*OCT4*, SSEA-4, teratomas	Data not shown	$380 per 10 mg $80 per six-well plate $100 per T75 $295 per T225 ***	~$10,000[b]	Fluorescence
StemAdhere[10] (recombinant E-cadherin)	mTeSR1	H1, H9, iPSC	>60	Accutase	*OCT3/4*, ZFP42, SSEA4, *NANOG*	H9 normal	$100 per kit $22 per 0.5 mg **	~$15,000[c]	Fluorescence
Peptide-SAM[44] (synthetic peptide)	mTeSR1 + ROCK	H1, H7, H9, H13, H14, IMR-90-1	6 (H1, H7), 19 (H9), 14 (H13), 17 (H14), 7 (IMR-90-1)	Manually	*OCT3/4*, ZFP42, SSEA4, *NANOG*	Trisomy on chromosome 17 (H14), other lines normal	Expensive *	>$15,000[d]	Fluorescence
Polymeric substrates									
PMVE-alt-MA[47]		HUES1, HUES9, iPSC	5	Accutase	*OCT4*, *NANOG*, teratomas	HUES1 and HUES normal	Inexpensive ***	~$1,100[e]	FTIR
PMEDSAH[42]		BG01, H9	3 (BG01) 10 (H9)	Mechanical	*OCT3/4*, SOX2 + EB (BG01), *OCT3/4*, SOX2, SSEA-4, TRA-1-60, TRA-1-81, teratomas (H9)	BG01 and H9 normal	Inexpensive ***	~$1,100[e]	WCA, XPS, FTIR, elastic modulus
APMAAm[43]	mTeSR1	H1, H9	>20	Collagenase	*OCT4*, SSEA-4	BG01 and WIBR3 normal	Inexpensive ***	~$1,100[e]	WCA, XPS
15A-30%[49]	mTeSR1 (pre-adsorption of WIBR3 HSA to polymer)	BG01, WIBR3	5	Collagenase	*OCT4*, SSEA-4, TRA-1-60, *NANOG* teratomas	BG01 and WIBR3 normal	Inexpensive ***	~$1,100[e]	WCA, elastic modulus, surface roughness, ToF-SIMS
Ultraviolet/ ozone treated TCPS	mTeSR1 + ROCK (pre-adsorption of hrVN, 20% HSA or 20% FBS to polymer)	BG01 WIBR1 WIBR3	>10	Collagenase accutase	*OCT4*, SSEA-4, SOX2, *NANOG*, teratomas	Normal >passage 5 with ROCK (abnormal at passage 5 without ROCK)	Inexpensive ***	~$1,000[f]	XPS, ToF-SIMS
HG21[51]	mTeSR1	RH1 H9	>20 (RH1) 9 (H9)	Thermally induced	*OCT3/4*, *NANOG*, SOX2, SSEA-4, TRA-1-60, EB, teratomas	Deletions/ duplications on chromosomes 8, 9, 13, 20 (passage 21)	Inexpensive ***	~$1,100[e]	XPS, rheology

[a]Scalability rating determined by the ability to synthesize the material in large quantities for hPSC production in a cost-effective and timely manner. *** is a highly scalable substrate and * is a substrate that is not scalable. [b]Estimated using 100 coated T75 flasks required to achieve 1 billion hPSCs. [c]Estimated from 150 kits required to coat a sufficient number of six-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 from the cost of commercial monomers of about $0.5 g^{-1} and 50 mg of dissolved polymer required to coat 350 T75 flasks. [f]Estimated from the cost of 350 T75 flasks. Polymer acronyms are defined in Fig. 1. EB, embryoid body; HSA, human serum albumin; TCPS, tissue-culture polystyrene; WCA, water contact angle; XPS, X-ray photoelectron spectroscopy; FTIR, Fourier transform infrared spectroscopy; ToF-SIMS, time-of-flight secondary-ion mass spectrometry.

the supportive peptide was required to achieve growth rates similar to Matrigel, with concentrations of BSP ranging from 0.75 to 1 mM, yielding 6–9 pmol mm^{-2} in peptide density. VN-PAS-coated flasks seeded with hPSCs and cultured for 4 days in defined medium showed uniform cell distributions and typical morphology, and expressed the pluripotency marker *OCT4*. This substrate has been developed commercially and marketed as Synthemax[9] (Table 2). Because of the biological components used in this substrate, however, it is expensive (about $100 for each T75 flask) compared with the widely used laboratory growth substrate Matrigel (approximately $15 per T75 flask). Another example of a growth substrate that has been commercially developed is StemAdhere[10]. This growth substrate uses 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. StemAdhere 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 cost of the cultureware alone to produce 1 billion hPSCs (an approximation for a single patient intervention) is estimated to be about $10,000 and $15,000 for Synthemax and StemAdhere, respectively (Table 3). This is likely to be prohibitive for cell expansion in clinical and biomedical uses, and is considerably greater than that of using Matrigel, which is estimated to be about $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 good manufacturing practice is still evolving for hPSC technologies; as more defined systems become available, they are likely to be required.

Polymer-based growth substrates

Systems that we classify as scalable are those that can be used to produce billions of cells in an economical and safe manner for many patients. A benchmark in the pharmaceutical industry to screen differentiated stem cells for drugs is to achieve a cost of less than $1 per well (in a 96-well plate). At present, this low cost is not achievable for pluripotent stem cell culture. The peptide–polymer-derived substrates mentioned previously are amenable to commercial development, but ideally the substrate should be fully synthetic using readily synthesized and cheap components. Polymers formed from readily synthesized monomers would be ideally suited to meet this challenge and emulate the success of treated polystyrene substrates used so widely for general cell culture.

To this end, a number of research groups have embarked on the search for polymeric substrates to maintain pluripotent stem cell expansion. Polymerization from surfaces has been used to prepare six acrylate-based surfaces through ozone-activation of tissue-culture polystyrene (TCPS) and subsequent surface-initiated polymerization with a range of acrylate monomers[42]. One of these materials (poly [2-(methacryloyloxy)ethyl dimethyl-(3-sulphopropyl) ammonium hydroxide]; PMEDSAH) was able to support the long-term culture of hPSCs in serum-free defined mTeSR1 medium (including protein supplement; Fig. 1d(iii)). H9 cells that were supported through 10 passages using StemPro medium showed normal karyotype, and expressed levels of pluripotency markers that were similar to cells cultured on Matrigel. However, no scalability was demonstrated using this substrate material. Another example of utlizing reactions with polystyrene is an aminopropylmethacrylamide-based coating (Fig. 1d(iv)) grafted to TCPS dishes using a photoinitiated addition polymerization[43]. H1 and H9-hOCT-pGZ hPSC lines were cultured in mTeSR1 medium for 10 passages, maintained typical cell morphology, and grew in colonies similar to Matrigel-cultured cells. Bovine serum albumin was proposed to play a crucial role in hPSC attachment achieved in the culture medium, and quartz crystal microbalance with dissipation experiments were used to identify the adsorption of bovine serum albumin to the growth substrate from the mTeSR1 medium.

High-throughput materials discovery for stem cell culture

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

Surface-modification strategies such as self-assembly have been used to present molecules capable of binding to cell-surface integrins with high spatial resolution[40]. Arrays of peptide-substituted alkanethiols have been prepared as SAMs on gold surfaces[44]. The molecules screened included peptides containing RGD- and glycosaminoglycan-binding epitopes, the most successful of these being a heparin-binding peptide derived from VN (GKKQRFRHRNRKG). This peptide was able to support long-term self-renewal of hPSCs at peptide densities of 0.5–25% (percentage of peptide-substituted alkanethiol in a mixed SAM monolayer) when combined with ROCK inhibitor or cyclic RGD peptide. The heparin-binding 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 using this peptide. However, hPSC expansion was not demonstrated over large areas, for instance in a culture flask.

Presynthesized polymer libraries have been printed as microarrays[45] using the concept of combinatorial polymer libraries first shown in 1997[46]. For high-throughput materials discovery, this has the limitation that polymer synthesis is time consuming, reducing the diversity of such arrays and slowing the follow up of leads generated using subsequent arrays. Ideally, the evolution from the initial screening to future-generation arrays arises from hypotheses being formed from a first-generation array, and subsequently tested in generations that evolve rapidly according to the results generated. In 2010, a polymer microarray consisting of 91 commercially available and presynthesized polymers was used to screen for hPSC attachment in StemPro medium[47]. A broad range of polymer backbones and side-chain functionalities were screened, including styrenes, acrylates and acrylamides spotted onto polyacrylamide-coated glass slides. Of the initial hits identified by high *OCT4* and *NANOG* expression using fluorescence microscopy to identify pluripotency, one polymer (poly(methyl vinyl ether-alt-maleic anhydride); Fig. 1d(ii)) was able to support hPSC attachment and self-renewal for more than five passages using StemPro medium. Scalable expansion of hPSCs on this polymeric substrate was not demonstrated beyond cell expansion on polymer-coated slides.

On-slide synthesis of polymer microarrays, achieved in 2004[48], allows rapid synthesis of acrylate polymers by combinatorial mixing of liquid monomers printed onto a hydrogel-coated slide before ultraviolet-photoinitiated free-radical polymerization. A strength of polymer microarrays is the ability to rapidly assess cell responses to a large polymer library coupled with surface analysis of the library on the array; this allows for interactions between the cells and material surface to be investigated.

Subsequently, several groups have used polymer microarrays to screen surface chemistries for hPSC attachment in a variety of culture media[47,48,50]. In 2010, several generations of polymer microarray were screened, starting with a library of 496 unique materials formed by mixing 16 acrylate 'major' monomers with six 'minor' monomers that were then contact-printed on a poly(2-hydroxyethyl methacrylate) (pHEMA)-coated substrate and polymerized *in situ* using ultraviolet irradiation[49]. Polymers with potential as supports for pluripotent stem cells were identified by their ability to support

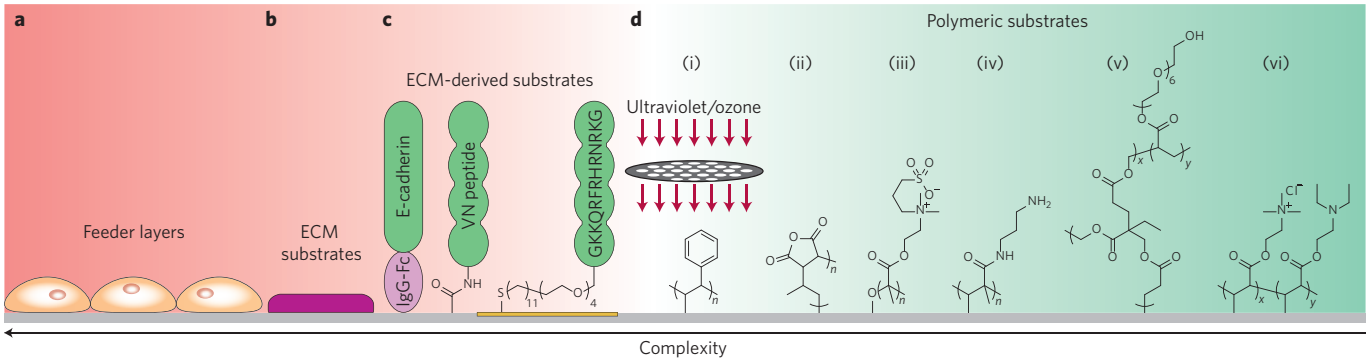

Figure 1 | The development of hPSC growth substrates. a, Feeder layers of MEFs support cell adhesion and condition the culture medium, with ECM proteins aiding hPSC self-renewal. **b**, Surface coating with an undefined ECM-protein-containing mixture such as Matrigel. **c**, Surface-immobilized functional epitopes of ECM components encourage hPSC attachment and self-renewal. **d**, Polymeric growth substrates provide an environment to adsorb essential ECM proteins from the culture medium: (i) ultraviolet/ozone-modified polystyrene, (ii) poly(methyl vinyl ether-alt-maleic anhydride), (iii) poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulphopropyl)ammonium hydroxide] (PMEDSAH), (iv) aminopropylmethacrylamide (APMAAm), (v) polymer microarray of triacrylate/diacrylate copolymer (15A-30%), and (vi) 2-(acryloyloxyethyl) trimethylammonium chloride/2-(diethylamino)ethyl acrylate copolymer (HG21).

the clonal growth of BG01-OCT4-GFP$^+$ cells from very low initial seeding densities over 7 days in MEF-CM (arrays preconditioned with FBS). High-content fluorescent microscopy was used to quantify cell responses to the individual polymer spots using OCT4-GFP. High-throughput surface characterization of the microarray was used to quantify the material chemistry and properties such as wettability and indentation elastic modulus, which were compared with cell performance 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. Surfaces pre-adsorbed with human VN 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 (more than five passages over 1 month) was reported on surfaces pre-incubated with human serum albumin (HSA), and scalability beyond microarray spots was not demonstrated for the hit materials (Fig. 1d(v)), although slides covered with the same polymer spots were used to obtain sufficient cell numbers for fluorescence-activated cell sorting (FACS) analysis.

A subsequent study used ultraviolet–ozone modification of polystyrene that maintained pluripotency for more than 10 passages on surfaces conditioned with HSA or human VN in mTESR1 medium[50]. 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 using this culture system (Fig. 1d(i)).

More recently, a microarray of 609 different thermoresponsive polymers produced by inkjet-printing 18 acrylate and acrylamide monomers with a crosslinker in various ratios was reported[51]. 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 (more than 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 step may be useful in automated systems for stem cell expansion. Growth rates of RH1 cells on hydrogel-coated coverslips were significantly lower than on Matrigel controls; RH1 cells took 8–10 days to reach 80% confluence on the hydrogel coating, as opposed to 4–5 days on Matrigel in mTESR1 medium. Xeno-free culture medium was not used, and the scalability of this growth substrate was not demonstrated beyond the coating of cover slips.

Mechanism of cell response to surface cues

Efforts have been directed towards understanding the effect of materials on the regulation of stem cell behaviour by designing substrates with particular chemistries, compliances, topographies, or containing biologically relevant moieties[44,52,53]. Cell-adhesion molecules that govern cell–matrix and cell–cell interactions play a crucial role in the long-term maintenance and self-renewal of adhered hPSCs. The identification of cell-surface integrins that can engage with Matrigel (β_1)- and VN ($\alpha_v\beta_3$ and $\alpha_v\beta_5$)-coated substrates has enabled understanding of how hPSC pluripotency is maintained[38,49,54,55]. Cell–cell interactions mediated by cadherins and their role towards hPSC behaviour have been extensively studied over recent years (see ref. 56 for a recent review of this area). Specifically, substrates presenting E-cadherin have been commercialized as StemAdhere, and have proven to be useful for hPSC expansion[10].

Adsorption of individual proteins on polymeric substrates and the subsequent effect on cellular performance have been well studied[57,58]. But knowing which proteins adsorb from protein-containing media onto materials, and the conformation they adopt on adsorption to a synthetic surface, is an essential element in gaining an understanding of material performance in protein-containing culture conditions.

The topography and elastic modulus of synthetic substrates have been shown to influence the differentiation of stem cells[59–62]. These studies highlight the importance of physical, as well as chemical, properties in regulating self-renewal and differentiation in future culture systems used for large-scale manufacture of hPSCs. Recently, the heparin-binding peptide GKKQRFRHRNRKG (see earlier discussion) was attached to hydrogel-based surfaces of various moduli to enable mechanical control of hPSC self-renewal[63]. Immobilization of the peptide on the hydrogel substrates was achieved by a chemoselective reaction between maleimide moieties and the peptide terminated with a cysteine residue. Only stiff hydrogels (10 kPa) were able to maintain hPSC pluripotency, which was aided by the activation of the Yes associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ). The hydrogel that exhibited a Young modulus of 10 kPa, determined by force-indentation measurements using atomic force microscopy, was most effective at inducing YAP/TAZ nuclear localization.

Modelling and predicting material performance

To support experimental materials discovery, computational methods capable of predicting the role of materials in encouraging cellular attachment have been explored[64] (Fig. 2). An early example of this was demonstrated for fibroblasts by identifying relationships between polymer properties and cell responses within a combinatorial library by using pre-synthesized copolymer-coated glass coverslips[65]. Linear correlations were observed between fibroblast proliferation and polymer-surface hydrophobicity for a subset of the library of polymers. Thermal and physical properties of the polymer library, such as glass transition temperature and water contact angle (WCA), were predicted successfully using a molecular descriptor called the total flexibility index (the number of carbon atoms at modification points within the copolymer structure). More recently, adhesion of human embryoid body cells to a library of polymers has been linked to molecular descriptors, indicating that computational approaches may be used to guide the design of materials production for experimentation with stem cells[66] (Fig. 2).

Surface chemical measurements can be used to determine the surface chemistries controlling cell attachment to materials, with statistical and machine-learning methods used to aid interpretation of large data sets. For example, 15 oxygen-containing plasma-deposited films were characterized using static secondary-ion mass spectrometry (SIMS)[67]. A correlation between the positive and negative secondary-ion spectra from the materials and endothelial cell growth was determined using multivariate partial least-squares (PLS) regression. The PLS model identified ions within the SIMS spectra that contributed towards high and low cell attachment. Using a development of this approach, a combination of high-throughput surface characterization (surface mass-spectral data) and multivariate analysis was used to predict the wettability of 576 polymers in a combinatorial microarray library[68,69]. A statistically valid PLS model between WCA measurements and spectra obtained using time-of-flight secondary-ion mass spectrometry (ToF-SIMS) was obtained. This study demonstrated the utility of multivariate analysis techniques such as PLS to model large data sets 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). Another study used PLS regression to explore the relationship between surface chemistry of a combinatorial polymer microarray and the colony-formation frequency of hPSCs[49]. Good agreement was found between the measured colony-formation frequency and that predicted from the ToF-SIMS spectra from the material surfaces, highlighting the importance of material surface chemistry in controlling stem cell response. The approach also helped to identify the controlling surface functionalities, to allow efficient materials discovery in which improvements from one microarray generation to the next are achieved to obtain the best-performing combination of materials from the library of monomers available. The best-performing polymers were pre-adsorbed with VN before cell seeding. To investigate the role of this step, ToF-SIMS of these protein-conditioned polymer surfaces was used to analyse the chemistry of the surface. Strong correlations between cell attachment and characteristic protein secondary ions were identified, indicating the synergy between the material's surface chemistry and the identity and amount of adsorbed proteins that enables colony formation. It is known from blocking experiments that the role of protein adsorption to polymeric growth substrates is pivotal in assisting hPSC attachment and self-renewal through engagement with cell-surface integrins[49]. Understanding

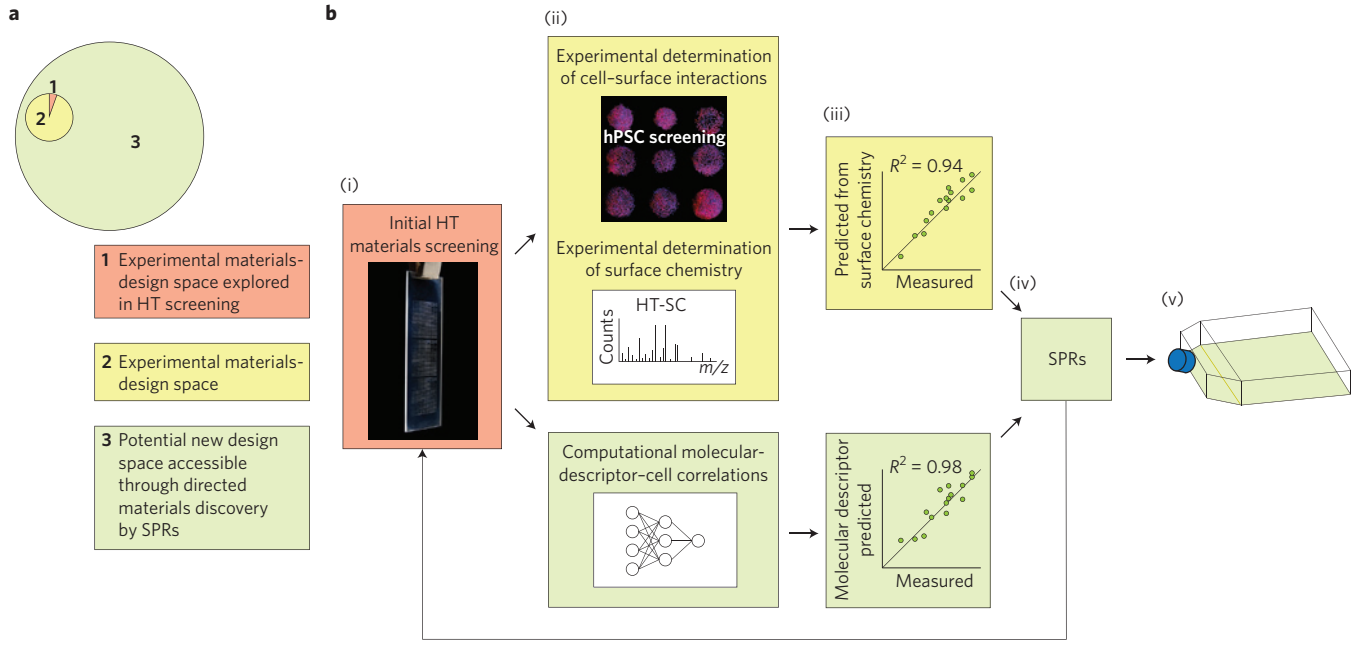

Figure 2 | High-throughput materials discovery. a, Concept. The small materials-design space that can readily be accessed experimentally (2), and the subset that can be explored in high-throughput screening (1), limits the discovery of new materials for hPSC culture. Directed materials discovery can be achieved through advanced modelling methods that enable structure–property relationships (SPRs) to be developed. This has the potential to allow researchers to access a materials-design space yet to be explored experimentally (3). HT, high throughput. **b**, Workflow. (i) High-throughput sample formats such as polymer microarrays can be screened for new growth substrates for hPSC culture to access a small proportion of the potential chemical space. (ii) High-throughput surface characterization (HT-SC) can be used in parallel to the biological assay. Correlation of the surface analytical data with cell performance using multivariate PLS regression links structure to function. Neural networks can identify molecular descriptors correlating with cell response. (iii) The predictive models have the potential to identify material chemistries that could not have been predicted from the experimental data alone. (iv) These advanced modelling techniques can be used to develop SPRs that can be used to explore a new materials-design space. This process can be performed in an iterative manner until an optimized lead candidate material has been found and scaled up to coated cultureware for hPSC expansion (v).

Box 2 | Challenges in hPSC culture for materials scientists.

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

One of the greatest challenges in materials design is the development of a fully synthetic growth substrate that can adsorb essential proteins in the desired conformation from the culture medium to aid 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. But there is a gap in knowledge about how biomolecules adsorb to synthetic substrates that 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: for instance, using sulphonated synthetic polymers to mimic the functional characteristics of heparin[42]. This hypothesis-driven approach has led to the development of polymer-based growth substrates that can achieve hPSC expansion in defined media[42,43,49,51].

Another design route for synthetic growth substrates is a combination of high-throughput materials discovery and computational screening. It is often not appreciated how vast is the possible 'space' of materials that could be synthesized, 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: the use of design of experiments (DoE) methods allows the number of experiments that are required to cover a given design space to be minimized[72]. Computational models of the data from these experiments then allow the properties of all materials within the design space to be predicted.

If large-scale high-throughput methods that capture sufficient molecular diversity on the materials can be developed, they should be capable of wide extrapolation into the 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 (Fig. 2).

the identity and conformation of the proteins that adsorb from complex protein-containing media to materials 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). At present, however, unequivocal identification of protein identity and conformation from complex-protein-containing media is not possible.

Outlook

The search for new materials for adherent hPSC culture has been greatly accelerated by the recent application of high-throughput sample-screening strategies such as polymer microarrays. Surface characterization and correlative and predictive models make this a powerful approach with which to search for new materials. This development of quantitative structure–property relationships (SPRs), by using the results from large experimental libraries linking polymer structure to hPSC performance on materials, is likely to broaden the chemical combinatorial space beyond what is currently explored to aid the search for better materials for hPSC culture. The materials-discovery process can be further aided by combining this with high-throughput screening of synthetic soluble factors that can replace biologically derived ingredients within hPSC culture media and increase the scalability of such culture systems.

High-throughput materials screening is moving towards directed discovery so as to allow exploration beyond the existing experimentally investigated chemical space, and towards utilizing experimentally determined surface structure–property relationships (sSPRs) and computationally determined molecular descriptors. Enabling these methods to evolve beyond the constraints of the current experimentally accessible chemical space will represent a step change in materials-discovery capabilities. Recently, molecular descriptors have been used to predict the response of stem cell attachment and to generate sSPRs *in silico* without the need for experimentally determined polymer characteristics[65]. Although the 'reverse SPR' problem (deducing a polymer structure from an SPR model) has been challenging in the past, developments in mathematics have recently provided practical methods for designing polymers with optimal properties from SPRs and molecular descriptors[69]. Success in this endeavour would open up the full range of materials to computational examination so as to direct synthesis efforts to potentially fruitful areas for experimental exploration. We expect that these and the other materials-discovery approaches covered in this Review will provide the materials necessary for the stem cell factories of the future.

Human pluripotent stem cells have presented 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 hPSCs needed (in the billions) to meet the demands of regenerative-medicine interventions currently in clinical trials. The long-term expansion and self-renewal of hPSCs in xeno-free and defined conditions is a prerequisite to achieving this. Xeno- and feeder-free E8 medium has now been commercialized[17], and E6 — a medium used to reprogramme somatic cells to hiPSCs before expansion in E8 — is currently under development. For adherent hPSC culture this will also need to be supported by chemically defined substrates that offer reproducibly high growth rates. Suspension hPSC culture has been shown as a promising alternative to adherent hPSC culture. Both refinement of the culture media components and improved growth rates compared with adherent systems will, however, 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 new polymers show promise; still, they largely require protein pre-adsorption, or display significant limitations in industrial scale-up (mainly cost). The make-up and functionality of the future systems required to address these challenges is still unknown. But several groups are taking clues from established large-scale industries. For example, the manufacturing

of semiconductors uses fully automated, closed-loop robotic systems to couple high-throughput production and quality-control parameters in the absence of human intervention. Combining synthetic materials able to support pluripotent stem cell expansion with such high-throughput processing methods will lead to stem cell factories in which large numbers of culture vessels will be used to expand cell numbers, for example slimline flasks using microfluidics to feed and quantify pluripotency status of adherent cells in fully automated, closed-loop systems. For this area to develop further, the concurrent development of pluripotency-compatible, next-generation substrates will be essential for economically viable hPSC manufacture. Substrates will probably include proprietary low-cost two-dimensional polymers, identified by high-throughput strategies, as well as three-dimensional configurations that allow switching between retention of pluripotency and induction of differentiation[71].

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Competing financial interests

The authors declare no competing financial interests.