# 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 (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. 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[1–4].

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[5], and human induced pluripotent stem cells (hiPSCs), derived by epigenetic reprogramming of somatic cells with stem cell-associated factors[6]. 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[7]. However, for biomedical applications to be realized, defined culture conditions need to be established in order to eliminate batch variability and xenogenic contaminants. Furthermore, scalable culture systems are required[8-9]. For adherent culture systems, scalability is often achieved by increasing the surface area of a candidate growth substrate in T-75 flasks (75 $cm^2$ growth area) without compromising biological performance as measured by pluripotency markers.

In comparison with a cell derived proteinaceous mixture such as Matrigel[10], polymers are not prone to batch-to-batch biological variations and they are readily amenable to large-scale manufacture; for example, injection moulding is currently used to form tens of millions of tissue-culture vessels per year. Consequently, there are significant efforts to identify synthetic substrates on which to support pluripotent stem cell expansion. 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 last 15 years towards more precisely defined culture systems for hPSCs. Initial reports of hPSC culture employed 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, transforming growth factor β (TGF-β) and Laminin-511 — that aid hPSC proliferation and self-renewal. In 1998, a MEF feeder layer was employed to support hPSC growth in a medium of 80% Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal bovine serum (FBS), 1mM glutamine, 0.1mM β-mercaptoethanol and 1% nonessential amino acids (NEAA)[5]. However, the use of non-human (xeno) feeder layers and animal-derived serum such as FBS represent a potential source of pathogens, such as endogenous retroviruses and xeno epitopes (such as nonhuman sialic acid and N-glycolneuraminic acid (Neu5Gc), a monosaccharide)[11]. Factors such as Neu5Gc can induce an immune response upon transplantation of hPSCs cultured using xenogenic methods, and limit their use to *in vitro* applications.

**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 relationships[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 using TeSR1 medium[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 Lamini 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 O3 medium and xeno-free H3 medium[43] |
| | Synthetic polymer surface employed in StemPro medium[47] |
| | Surface chemistry of polymer surfaces shown to influence hESC growth[53] |

| | | |
|---|---|---|
| 2011 | Long-term hESC self-renewal on polymer surface achieved in mTeSR1 medium[48] |
| 2012 | Human embryoid body cell adhesion to a combinatorial polymer library modelled using molecular descriptors[70] |
| | Long-term self-renewal of hPSCs on Laminin E8 surfaces in TeSR2 medium[42] |
| 2013 | Long-term hESC self-renewal and thermally-triggered passaging achieved using thermoresponsive hydrogel in mTeSR1 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 biological substrates 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]. 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 make understanding and controlling the cell/material interface more difficult, 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)[11, 13-24]. Subsequently, Matrigel was offered as an LDV-free product.

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, 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[25]. 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™, Liquid Thermo Scientific[15] | ✓ | ✓ | ✓ | Plasma treated polystyrene | Cultureware |
| | CellStart™, Invitrogen[16] | ✓ | ✓ | ✓ | Humanized protein mixture | Liquid |

| | | Defined | Feeder-free | Xeno-free | Serum-free |
|---|---|---|---|---|---|
| Culture Medium | HEScGRO, Millipore | ✓ | × | ✓ | ✓ |
| | mTeSR1™, STEMCELL Technologies[17] | ✓ | ✓ | × | ✓ |
| | TeSR2™, STEMCELL Technologies[17] | ✓ | ✓ | ✓ | ✓ |
| | StemPro™, Invitrogen[18] | ✓ | ✓ | × | ✓ |
| | NutriStem™, STEMGENT[19] | ✓ | ✓ | ✓ | ✓ |
| | E8™, GIBCO[20] | ✓ | ✓ | ✓ | ✓ |
| | XVIVO™ 10, Lonza[21-22] | ✓ | ✓ | ✓ | ✓ |
| | RegES[23] | ✓ | × | ✓ | ✓ |
| | hESF9[24] | ✓ | ✓ | ✓ | ✓ |

A major milestone in hPSC culture was the development of a defined culture medium called TeSR1, and published in 2006 (ref. 17). The essential ingredients within the TeSR1 medium were the protein basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGFβ), Lithium Chloride, γ-aminobutyric acid (GABA) 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 vitronectin. The derivation of two new hPSC lines, WA15 and WA16, was also achieved using TeSR1. However, WA15 became trisomic (three chromosomes as opposed to two) for chromosome 12 between 4 and 7 months in culture. A variant of the TeSR1 culture cocktail has been commercialized as mTeSR1, employing BSA and zebrafish bFGF, as a cheaper alternative to the xeno-free culture medium. To further aid hPSC culture, supplements such as Rho-associated kinase (ROCK) inhibitors have been employed to reduce dissociation-induced cell apoptosis when working in defined medium[26]. However 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 that identified small molecules added to the culture media promoted hPSC survival after trypsin dissociation from a Matrigel substrate[27]. Thiazovivin (Tzv) and Pyrintegrin (Ptn) were both found to dramatically increase cell survival versus DMSO controls. High-throughput screening of small molecules can rapidly identify essential ingredients within current culture media employed in hPSC culture, and be used to reduce the number of components within the hPSC culture system (Box 1; refs. 26–40). Furthermore, this screening approach can identify novel culture medium supplements to aid the survival and self-renewal of hPSCs in defined culture conditions.

**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. Currently, the definition of GMP in terms of hPSC technologies is still being established as undefined culture systems have entered phase 1 clinical trials. However, with the advent of defined substrates, 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 major challenge in hPSC culture to 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 most commonly consist of biological molecules such as growth factors and serum proteins to aid successful hPSC culture. 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 (Table B1).

These molecules can manipulate hPSC activities by the activation or inhibition of key molecules within signalling pathways including MEK (PD0325901 and PD98059)[28-29], ROCK (Y27632, Thiazovivin, HA-1077, and Pinacidil)[26-27,30-31], FGF (SU5402 and PD173074)[32], ERK (SC1)[33], and GSK3 (Bio, CHIR99021, and Bisindolylmaleimide 1i)[28,34-35]. As such, small molecule chemistries hold great potential as medium supplements in the development of fully defined and cost-effective hPSC culture conditions that are amenable to GMP scale-up.

**Table B1. Small molecule supplements for hPSC survival and self-renewal.**

BIO (6-bromoindirubin-3-oxime)[34]

Bisindolylmaleimide 1i[35]

CHIR99021 (Calbiochem)[28]

Geldanamycin[36]

HA-1077 (Fasudil)[30]

ID-8[37]

| |
|---|
| PD0325901[28] |
| PD173074[32] |
| PD98059[29] |
| Pinacidil[31] |
| Pyrintegrin[27] |
| Retinol[38] |
| SC1 (Pluripotin)[33] |
| SU5402[32] |
| SU6656[40] |
| Thiazovivin[27] |
| U0126[39] |
| ROCK Y-27632[26] |

**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, -332 and -511 were identified 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.

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)[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 α6β1 integrin. This phenomenon was thought to have aided the long-term self-renewal of the hPSCs. However, passaging the cells required physical removal of the cells from the LN-511 coated plates, resulting in cell clumps rather than single-cell suspensions. Furthermore, this method of passaging is incompatible with automation which 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, substrates presenting specific peptide sequences have been developed to identify and utilize specific interactions at the cell/material interface that mediate stem cell behaviour. Microarrays of spotted 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 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)[45]. Of the six peptides employed, 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 the supportive peptide was required to achieve growth rates similar to Matrigel, with concentrations of BSP ranging from 0.75–1 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[13] (Table 2). However, because of the biological components used in this substrate, it is expensive (about $100 for each T75 flask) compared to 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[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. 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 culture ware alone to produce 1 billion hPSCs (an approximation for a single patient intervention) are 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 (GMP) is still evolving for hPSC technologies; as more defined systems become available, they are likely to be required.

**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[13] (peptide-polymer conjugate) | X-VIVO 10, 80ng/mL hrbFGF 0.5ng/mL hrTGF-β | H1, H7. | 10 | collagenase | OCT4, SSEA-4, 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] | Fluorescence |
| Peptide-SAM[49] (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 | OCT4, SSEA-1/3/4, SOX2, TRA-1-60, TRA-1-81, teratomas | Trisomy on chromosome 17 (H14), other lines normal | Expensive * | >$15,000[d] | Fluorescence |
| **Polymeric Substrates** | | | | | | | | | |
| PMVE-alt-MA[46] | StemPro + suppl. with DMEM-F12 (2% (v/v) BSA) | HUES1, HUES9 iPSC | 5 | accutase | OCT4, NANOG teratomas | HUES1 and HUES9 normal | Inexpensive *** | ~$1,100[e] | FT-IR |
| PMEDSAH[47] | StemPro suppl. with DMEM-F12 + L-glutamine, 15mM HEPES | 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, FT-IR elastic modulus |
| APMAAm[48] | mTeSR1 | H1, H9 | >20 | collagenase | OCT4, SSEA-4 + EB | H1 and H9 normal (p10) | Inexpensive *** | ~$1,100[e] | WCA, XPS |
| 15A-30%[53] | mTeSR1 (pre-adsorption of 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 |
| UV/Ozone treated TCPS | mTeSR1 + ROCK (pre-adsroption of hrVN, 20% HSA or 20% FBS to polymer) | BG01, WIBR1 WIBR3 | >10 | collagenase accutase | OCT4, SSEA-4, SOX2, NANOG, teratomas | normal >p5 with ROCK (abnormal at p5 without ROCK | Inexpensive *** | ~$1,000[f] | XPS, ToF-SIMS |
| HG21[55] | 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 (p21) | Inexpensive *** | ~$1,100[e] | XPS, rheology |

[a] Scalability determined by the ability to synthesize the material in large quantities for 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 about $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. 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). This is not currently 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 synthesised 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 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 *via* ozone-activation of tissue culture polystyrene (TCPS) and subsequent surface-initiated polymerization with a range of acrylate monomers[47]. One of these materials (poly [2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide]; PMEDSAH), was able to support the long-term culture of hPSC in serum-free defined mTeSR1 medium (including protein supplement; Fig. 1d(iii)). H9 cells 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 reactions with polystyrene is a aminopropylmethacrylamide-based coating (Fig. 1d(iv)) grafted to TCPS dishes using a photoinitiated addition polymerization[48]. 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 (BSA) was proposed to play a crucial role in hPSC attachment achieved in the culture medium. Quartz crystal microbalance with dissipation (QCM-D) experiments were used to identify BSA adsorption to the growth substrate from the mTeSR1 medium.

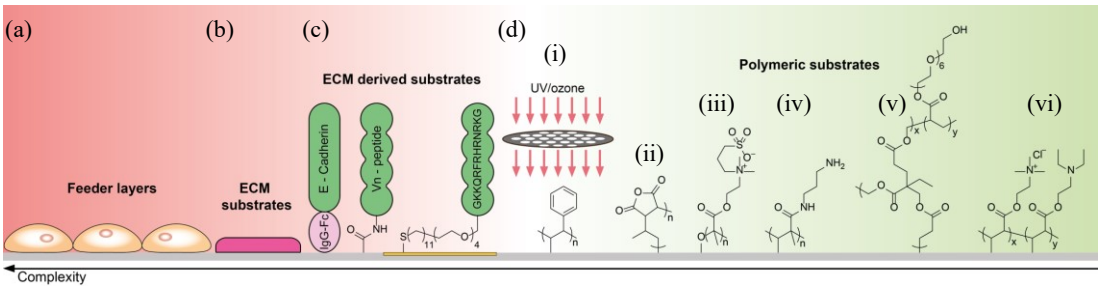


**Figure 1 | The development of hPSC growth substrates.** (a) Feeder layers of MEFs to support cell adhesion and to condition the culture medium with ECM proteins to facilitate hPSC self-renewal. (b) Surface coating with an undefined ECM protein-containing mixture such as Matrigel. (c) Functional epitopes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

## 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 of 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 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[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, for instance in a culture flask.

Pre-synthesized polymer libraries have been printed as microarrays[50], employing the concept of combinatorial polymer libraries first shown in 1997 (ref. 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 follow up of leads generated using subsequent arrays. Ideally, the evolution from the initial screen to future generation arrays arises from hypotheses being formed from a first generation array that are subsequently tested in generations that evolve rapidly according to the results generated. In 2010, a polymer microarray consisting of 91 commercially available and pre-synthesized polymers was employed to screen for hPSC attachment in StemPro medium[46]. A broad range of polymer backbones and side-chain functionalities were screened, including styrenes, acrylates and acrylamides spotted onto acrylamide-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 5 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 (ref. 52), allows rapid synthesis of acrylate polymers by combinatorial mixing of liquid monomers printed onto a hydrogel-coated slide prior to UV photoinitiated free-radical polymerization. A strength of polymer microarrays is the ability to rapidly assess cell response to a large polymer library coupled with surface analysis of the library on the array, to allow cell–material surface interactions to be investigated[3].

Subsequently, several groups have used polymer microarrays to screen surface chemistries for hPSC attachment in a variety of culture media[46,52,54]. In 2010, a number of generations of polymer microarray were screened, starting with a library of 496 unique materials formed by mixing 16 acrylate ‘major’ monomers with 6 ‘minor’

monomers that were then contact printed onto a poly(2-hydroxyethyl methacrylate) (pHEMA)-coated substrate and polymerized *in-situ* using UV irradiation[53]. Polymers with potential as supports for pluripotent stem cells were identified by their ability to support the clonal growth of BG01-OCT4-GFP$^+$ cells from very low initial seeding densities over 7 days in MEF-CM (arrays pre-conditioned with FBS). High-content fluorescent microscopy was used to quantify cell response to the individual polymer spots using OCT4 GFP. High-throughput surface characterization of the microarray was used to quantify the materials chemistry and properties such as wettability and indentation elastic modulus, which were compared to 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. 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 (more than 5 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 laser scanning cytometry.

A subsequent study used UV-ozone modification of polystyrene which maintained pluripotency for more than 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 (Fig. 1d (i)).

More recently, a microarray of 609 different thermoresponsive polymers produced by ink-jet printing 18 acrylate and acrylamide monomers with a crosslinker in various ratios was reported[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 (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$^{o}$C for 30 minutes. This step may be useful in automated stem-cell-expansion systems. Growth rates of RH1 cells on hydrogel-coated coverslips were significantly lower than Matrigel controls; RH1 cells took 8–10 days to reach 80% confluence on the hydrogel coating, as opposed 4–5 days on Matrigel in mTESR1 medium. Xeno-free culture medium was not used, and scalability of this growth substrate was not demonstrated beyond coating of cover slips. The thermo switch of the gels was characterized by bulk rheology measurements of materials on 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[49,56-57]. 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 ($\beta_1$) and Vitronectin ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) -coated substrates has enabled understanding of how hPSC pluripotency is maintained[42,53,58-59]. Cell–cell interactions mediated by cadherins and their role towards hPSC behaviour have been extensively studied over recent years (see ref. 60 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[14].

Adsorption of individual proteins on polymeric substrates and the subsequent effect upon cellular performance have been well studied[61,62]. However, 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. Such knowledge will ultimately direct materials discovery towards improved substrates for hPSC culture.

The topography and elastic modulus of synthetic substrates have been shown to influence the differentiation of stem cells[63–65]. 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[67]. 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 facilitated by the activation of the Yes-Associated Protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ). The hydrogel which exhibited a Young Modulus of 10 kPa, determined by force indentation measurements using AFM, 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[68] (Fig. 2). An early example of this was demonstrated for fibroblasts by identifying polymer–cell-response relationships within a combinatorial library *via* pre-synthesized copolymer-coated glass coverslips[69]. 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 ($T_g$) 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, human-embryoid-body cell adhesion 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 (Fig. 2)[70].

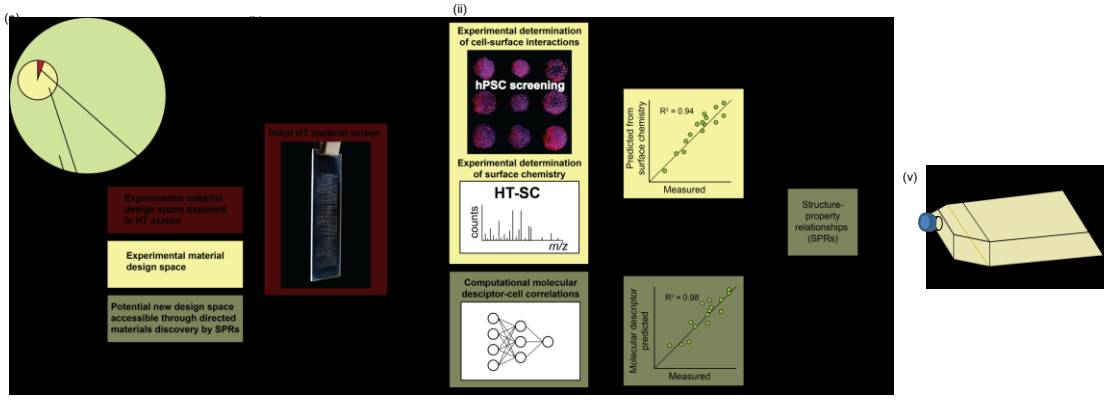


**Figure 2 | High-throughput materials discovery.** (a) **Concept**: The small materials-design space that can readily be accessed experimentally limits the discovery of new materials for hPSC culture. Directed materials discovery can be achieved through advanced modelling methods that enable structure-property relationships (SPRs) to be developed. This has the potential to allow access to a materials design space yet to be explored experimentally. (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 can be employed in parallel to the biological assay. Correlation of the surface analytical data with cell performance using multivariate partial least-squares (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 culture ware for hPSC expansion (v).

Surface chemical measurements can be used to determine the surface chemistries controlling cell attachment to materials, with the aid of statistical and machine-learning methods employed to aid interpretation of large data sets. For example, 15 oxygen-containing plasma-deposited films were characterized using static secondary ion mass spectrometry (SIMS)[71]. 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 (MVA) was employed to predict the wettability of 576 polymers in a combinatorial microarray library[72-73]. 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 MVA techniques such as PLS to model large 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). Another study 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 material surface chemistry in controlling stem cell response. The approach also helped identify the controlling surface functionalities, to allow efficient materials discovery in which improvements from one micro array 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 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 characteristic protein secondary ions were identified, indicating the synergy existing between the material's surface chemistry and the identity and amount of 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). At present, however, 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 scalable 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[74]. 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**).

## 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. Development of quantitative structure–property relationships (SPRs), by using the results from large experimental libraries linking polymer structure to hPSC performance on materials, are likely to broaden the chemical combinatorial space beyond what is currently explored to facilitate the search for better materials for hPSC culture. The materials-discovery process can be further aided through a combination 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 the ability to carry out *directed high-throughput materials 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. To enable 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[70]. Although the ‘reverse SPR’ problem (backing out 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[75]. 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 anticipate that these and the other material-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- 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, both 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; still, they largely require protein pre-adsorption, or display significant limitations in industrial scale up (mainly cost). What the make-up and functionality will be of the future systems required to address these challenges is still unknown. However, several groups are taking clues from established large scale industry. For example, manufacturing of semiconductors uses fully automated closed 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 enable the stem cell factories of the future in which large numbers of culture vessels will be used to expand cell numbers, e.g. 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 likely include proprietary low cost 2D polymers, identified by high-throughput strategies, as well as 3D configurations that allow switching between induction of pluripotency and differentiation[76].

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## Competing Financial Interests

The authors declare no competing financial interests.

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