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Feature article

Synthetic substrates for long-term stem cell culture

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ABSTRACT

Stem cells have a host of applications in regenerative medicine and basic research. However, clinical translation hinges on the availability of effective stem cell expansion. Stem cell expansion has been limited due to the use of xenogenic factors in the culture system, batch-to-batch variation, and processes that do not readily lend themselves to scale-up. Synthetic substrates represent attractive alternatives to standard feeder layer culture, as they address many of these pressing limitations. Specifically, we use a grafting-to approach to create a zwitterionic hydrogel capable of maintaining human pluripotent stem cells in long-term culture. This approach enables the control of substrate physiochemical properties, is relatively inexpensive, and results in a substrate with good storage and sterilization stability. In this feature, we focus on the contributions of our culture system to prolonged stem cell culture and compare it to other culture systems currently available.

1. Introduction

The cellular environment is complex and plays an important role in cellular properties and processes [1]. For example, environmental cues have been shown to influence cell adhesion, proliferation, and gene expression [2,3]. As a result, cell–material interactions are an integral part of *in vitro* cell studies and investigators have used synthetic biomaterials to mimic the cellular microenvironment in terms of its physiochemical properties [4,5]. However, the influence of a single substrate property, such as surface chemistry, wettability, and roughness, is difficult to assess, because these properties may be interconnected and work synergistically to garner a particular cellular response [6]. In addition to proliferation and gene expression, environmental cues also impact the differentiation of stem cells [7–9]. Human pluripotent stem cells (hPSCs), due to their ability to differentiate into multiple cell types, have a myriad of applications in the healthcare industry [10,11]. Potential uses include cures/treatments for diseases such as heart failure and diabetes as well as drug safety and efficacy testing [12–14].

Feeder layers of mouse embryonic fibroblasts (mEFs) and Matrigel™, an undefined gelatinous protein mixture secreted by mouse carcinoma cells, are currently utilized in hPSC culture [15]. Matrigel™ is typically placed on the cell culture substrate prior to cell seeding in order to generate a thin monolayer that serves as a complex, extracellular matrix (ECM). Although hPSCs show therapeutic promise, one of the major hurdles inhibiting clinical adoption is the use of this Matrigel™ culture system as it results in batch-to-batch variation, uses xenogenous factors which warrant immunogenic concerns, and is not suitable for large-scale hPSC expansion [16]. Synthetic substrates are a promising alternative for prolonged hPSC culture as they address many of these issues. Because synthetic surfaces are generated from defined materials and processes, there is little variation between batches and thus has greater potential for scale-up [17]. More importantly, eliminating the need for animal byproducts eradicates concerns regarding immunogenicity [18].

This feature article highlights new developments in the use of synthetic biomaterials as substrates in the long-term culture of hPSCs. In particular, a zwitterionic polymeric system developed in our lab is described in terms of its fabrication, efficacy, and stability. This synthetic substrate is then compared to synthetic culture systems generated by other research groups that have demonstrated efficacy in maintaining prolonged hPSC culture. In this

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instance, substrates are assessed in terms of desired criteria for hPSC culture systems and provide a framework for examining challenges in the design and fabrication of substrates for expanding hPSCs.

2. Hydrogels and stem cells

In an attempt to replace poorly defined biological matrices, numerous materials have been studied as potential stem cell substrates including electroactive polymers, tissue culture plastic, and self-assembled monolayers of alkanethiols [19–21]. Recent work in our group has focused on the use of hydrogels as a platform for stem cell culture. To that end, synthetic cell culture substrates are generated via surface-initiated graft polymerization of chemically defined polymers. In particular, various methacrylates were grafted onto tissue culture polystyrene (TCPS) dishes. Synthesis occurs in an oxygen-free glass reaction vessel. Briefly, the reaction vessel is degassed via a vacuum-argon purge cycle, which is completed three times. Simultaneously, the solvent, comprised of ethanol and deionized water in a volumetric ratio of 1:4, is degassed via vacuum for 40 min. Then the degassed solvent and the monomer of interest are added to the reaction vessel and heated such that the temperature range is between 76 and 82 °C. Tight temperature control is important for effective polymerization. Prior to monomer synthesis, free radicals must be created on the TCPS dishes by UV ozone plasma treatment. Later, these free radicals will enable the polymerization of the monomers of interest. Once the reaction has reached the desired temperature, TCPS dishes are added to the reaction vessel and polymerization proceeds for 2.5 h (Fig. 1).

After the reaction is complete and the reaction vessel has been cooled to at least 60 °C, the dishes are removed and rinsed overnight in a 1% saline solution that is maintained at 50 °C to remove any excess monomer. Subsequent rinsing steps with 1% saline and deionized water ensure that any unreacted monomer has been eliminated [23]. Because the influence of hydrogel structure on hESCs was unknown, a total of six methacrylate derivatives were generated for cell screening. Specifically, poly[carboxybetaine methacrylate] (PCBMA), poly[[2-(methacryloyloxy)ethyl]trimethylammonium chloride] (PMETAC), poly[poly(ethylene glycol) methyl ether methacrylate] (PPEGMA), poly[2-hydroxyethyl methacrylate] (PHEMA), poly[3-sulfopropyl methacrylate] (PSPMA), and poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] (PMEDSAH) were characterized in terms of their material properties and by the ability of hESCs to adhere and maintain an undifferentiated state on these surfaces (Fig. 2) [22].

Of the hydrogels assessed, only the zwitterionic PMEDSAH was able to support undifferentiated hESCs from two cell lines (BG01 and H9) for long-term passage (passage number $\geq$25) as indicated by hESC gene expression, karyotype, and embryoid body formation (Fig. 3). Results from the synthetic substrates were compared to Matrigel™, which served as a control and no significant difference was noted. As a proof of concept, a commonly utilized media from animal-derived products, mouse embryonic fibroblast-conditioned media (MEF-CM), was used in this initial screening study. However, since the ultimate goal of our work is to make a more chemically defined system with enhanced clinical relevance, subsequent studies focused on the behavior of hESCs on PMEDSAH-coated dishes using media which lacked non-human animal products or in serum-free, defined media.

2.1. Storage and sterility of PMEDSAH coatings

Maintenance of material properties after prolonged storage and exposure to common sterilization methods are important aspects of making synthetic stem cell substrates commercially viable. The stability of the PMEDSAH coating was investigated in an accelerated six-week storage study. In this instance, PMEDSAH-coated dishes were stored at three different conditions (all at room temperature) – (1) ambient, (2) inert (in a glove-bag under nitrogen atmosphere) and (3) vacuum (in a desiccator attached to a vacuum pump). Fourier transform infrared spectroscopy (FTIR) and/or X-ray photoelectron spectroscopy (XPS) was used to assess the stability of the film. Elemental analysis of XPS samples exposed to the various storage conditions after six weeks were compared to those values previously reported in literature (Table 1) [22].

XPS indicates little difference between samples stored under air and nitrogen. However, vacuum conditions cause slight differences in the overall composition. FTIR spectroscopy of samples stored under ambient conditions (in air at room temperature) revealed characteristic bands at 1732.9 cm^{-1} and 1208.4 cm^{-1} for carbonyl and sulfonate groups (Fig. 4). Characteristic stretches were readily apparent in air and thus a difference under inert conditions is not expected.

The compatibility of PMEDSAH-coated dishes towards common sterilization methods, particularly e-beam- and gamma-radiation, was also investigated. E-beam- and gamma-radiation were selected because these methods are widely accepted for the sterilization of biomedical products, and unlike ethylene oxide treatments, no residual chemicals are left behind after processing [24]. PMEDSAH-coated dishes were exposed to three different levels of e-beam-radiation (10, 20 and 50 kGy) and gamma-radiation (8–15, 22–40, 45–75 kGy) respectively. The effect of radiation on the coatings was then investigated by XPS and FTIR. The material composition of samples after radiation was compared to the values previously reported in literature (Table 2) [22].

Overall, XPS indicates that radiation does not cause a significant difference in the PMEDSAH coatings. This is further confirmed by FTIR analysis as characteristic carbonyl, 1732.9 cm^{-1}, and sulfonate, 1208.4 cm^{-1}, bands are evident for each radiation type and level (Fig. 5).

2.2. PMEDSAH and human embryonic stem cells in xenofree/defined media

We further extended our work by culturing BG01 and H9 cells on PMEDSAH-coated dishes using a commercially available xeno-free media, or media that lacks non-human animal products, but was instead conditioned with human cell serum. Matrigel™ was also used as a control. PMEDSAH substrates were shown to

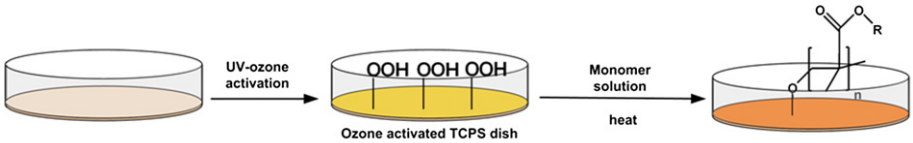
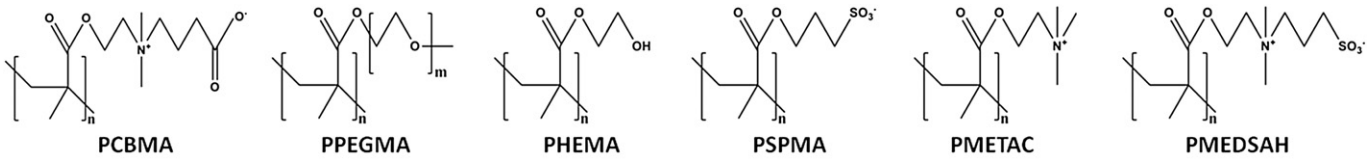


Fig. 1. Schematic depicting the graft-polymerization process used to fabricate the polymer coatings. UV ozone was utilized to activate the tissue culture polystyrene dishes and then methacrylate-based monomers were subsequently polymerized on the surface. Reproduced from Ref. [22] with permission.



	PCBMA	PPEGMA	PHEMA	PSPMA	PMETAC	PMEDSAH
Contact Angle	71.6 ± 4.8	63.3 ± 3.1	56.0 ± 3.7	50.2 ± 4.1	40.5 ± 5.7	17.1 ± 1.1
Reduced Elastic Modulus (GPa)	3.3 ± 0.1	3.9 ± 0.1	3.0 ± 0.5	3.0 ± 0.1	3.3 ± 0.1	2.5 ± 0.1
Cell-aggregate adhesion (%)	0	5 ± 1	12 ± 1	14 ± 2	8 ± 1	15 ± 1
Number of passages	0	1	2	2	2	25

Fig. 2. Long-term culture of H9 hESCs on methacrylate-derivative coatings with mouse embryonic fibroblast (MEF)-conditioned media. Table provides information about substrate properties (contact angle, reduced elastic modulus (GPa) (mean ± s.d.)) and cell behavior (initial hESC aggregate adhesion (mean ± s.e.m.) and number of passages achieved) on each polymer coating. Reproduced from [22] with permission.

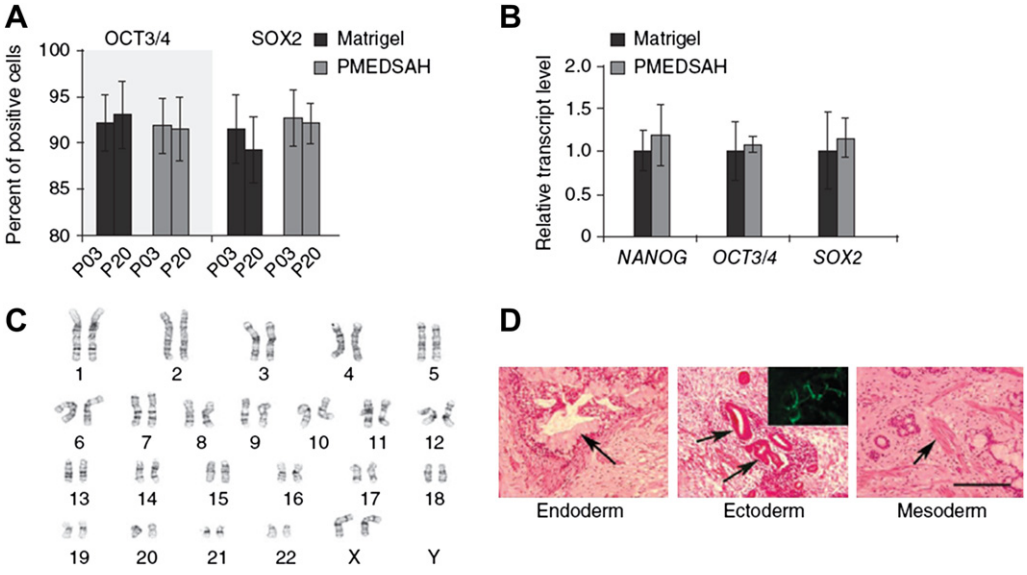


Fig. 3. Cellular characterization of hESCs cultured on PMEDSAH substrates in MEF-CM. (A) Percentage (mean ± s.e.m.) of hESCs expressing OCT3/4 and SOX2 at passages 3 (P03) and 20 (P20). (B) Relative transcript levels of *NANOG*, *OCT3/4* and *SOX2* from hESCs cultured on PMEDSAH and Matrigel™. (C, D) After 25 passages, hESCs cultured on PMEDSAH (C) maintained a normal karyotype and (D) retained pluripotency as demonstrated by teratoma formation in immunosuppressed mice. Hematoxylin and eosin-stained paraffin sections indicating endoderm (goblet-like cells at arrow), ectoderm (neuroepithelial aggregates at arrow; and cells expressing neuron-restricted protein β-III tubulin in inset) and mesodermal derivatives (cartilage, connective tissue and muscle at arrow). Scale bar, 200 μm. Reproduced from [22] with permission.

maintain pluripotency through 15 passages as evidenced by cell-population doubling times, hESC cell markers, and karyotyping. Not only were these culture conditions more clinically relevant in terms of the media utilized, but they also resulted in enhanced cell adhesion; at least for one of the hESC lines. In particular, hESC aggregate adhesion for H9 cells was significantly higher at each passage for cells grown on PMEDSAH in human cell-conditioned media (hCCM), 86 ± 6%, than in MEF-conditioned media, 15 ± 2%, (Fig. 6).

Table 1
XPS of PMEDSAH-coated substrates after 6 weeks of storage under various environmental conditions, as compared to samples that were not stored.

Storage conditions			
Element	Experimental		
	Air	Vaccum	Nitrogen
C 1s	74.1	79.4	74.6
O 1s	17.3	18.3	20.8
N 1s	3.8	0.9	1.8
S2p	4.8	1.4	2.8
Element	Theoretical	From nature biotech	
C 1s	61.1	72.7	
O 1s	27.8	20.9	
N 1s	5.6	3.2	
S2p	5.6	3.2	

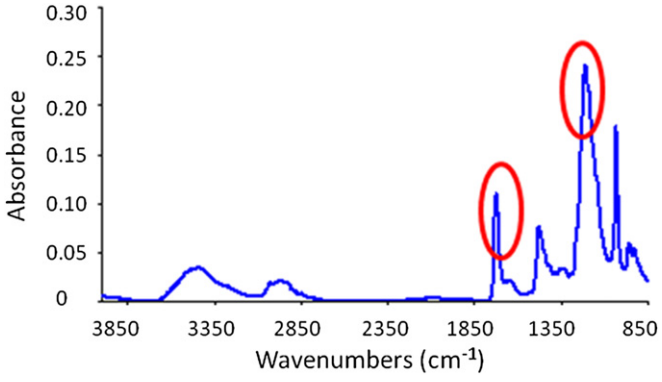


Fig. 4. FTIR of PMEDSAH coatings stored for six weeks under ambient conditions.

Table 2
XPS of PMEDSAH-coated substrates after batch sterilization with either e-beam- or gamma-radiation as compared to unsterilized samples.

E-beam			
Element	Experimental		
	10 kGy	25 kGy	50 kGy
C 1s	73.2	72.9	67.3
0 1s	20.8	21.4	23
N 1S	2.6	2.1	3.9
S 2p	3.5	3.7	5.9
Gamma			
Element	Experimental		
	8–15 kGy	22–40 kGy	45–75 kGy
C 1s	70	71.6	70.6
0 1s	21.6	21.5	21.8
N 1s	3.6	3.4	3.2
S 2p	4.7	3.5	4.5
Element	Theoretical		From nature biotech
C 1s	61.1		72.7
O 1s	27.8		20.9
N 1s	5.6		3.2
S 2p	5.6		3.2

Finally, we explored the ability of PMEDSAH-coated substrates to promote the undifferentiated growth of hESCs in defined media conditions using two commercially available serum-free media, StemPro™ and mTeSR™. Attempts to passage H9 hESCs on PMEDSAH-coated dishes in mTeSR™ were unsuccessful and the focus was shifted to the StemPro™ media. These culture conditions led to the successful proliferation of undifferentiated H9 cells on PMEDSAH substrates throughout 10 passages while BG01 cells were maintained for 3 passages. The undifferentiated nature of the H9 cells was confirmed via staining for hESC pluripotency markers and the directed differentiation of the cells into three specific lineages as specified by immunofluorescence and gene expression (Fig. 7). In subsequent culture, pluripotency could be maintained for up to 25 passages under these conditions.

3. Emergence of alternative synthetic culture systems and benchmarking

Due to the limitations associated with undefined matrices, more defined stem cell culture systems have been investigated. After our initial publication based on PMEDSAH [22], other synthetic polymers have shown the ability to maintain stem cells in the undifferentiated state for numerous passages including another methacrylate containing polymer, hit 9, and an anhydride containing polymer poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) (Fig. 8) [25,26]. Hit 9 is fabricated through the use of photopolymerization while PMVE-alt-MA is generated by free radical polymerization. The aforementioned systems are entirely polymeric, but materials that have attached biomolecules, such as peptides, have also been identified. In particular, Synthemax™ (Corning) consists of an acrylate polymer with covalently immobilized amine-containing peptides on the surface (Fig. 8) [27]. Polymers are not the only materials of choice, as GKKQRFRHRNRKG, a heparin-binding peptide, has been conjugated to self-assembled alkanethiol monolayers and subsequently utilized for stem cell culture (Fig. 8) [28]. Synthetic biomolecules such as recombinant proteins and synthetic peptides have also served as substrates in cell culture. For example, a human recombinant protein, specifically laminin-511, has been generated to facilitate long-term hPSC culture (Fig. 8) [29]. Furthermore, synthetic peptides designed to engage specific integrins involved in cell adhesion have been evaluated for hESC culture and initial results demonstrate their ability to support hESC adhesion and proliferation, though efficacy for prolonged culture has yet to be realized [30]. Creating new materials is not the only approach to generating synthetic culture systems. Polystyrene is a commonly used tissue culture plastic and it may be augmented to enhance prolonged stem cell culture. Recently, Saha et al., exposed polystyrene surfaces to short-wavelength UV in different doses to generate distinct surface chemistries [31]. Surfaces displaying particular amounts of carboxylic acid/ester and nitrogen-containing moieties were shown to promote long-term culture of pluripotent stem cells.

The ideal stem cell culture platform would support long-term expansion (≥20 passages) of undifferentiated stem cells, maintain efficacy in defined/xenofree media, has compatibility with common sterilization techniques, results from a process that is scalable, reusable, relatively inexpensive, and demonstrates efficacy for multiple stem cell lines and types [32–34]. Though several strategies other than grafting of PMEDSAH exist for prolonged stem cell culture, they are not without their limitations. In particular, PMEDSAH, Synthemax™, GKKQRFRHRNRKG, and recombinant laminin-511 have demonstrated efficacy for 10 or more passages of hESC culture in xenofree/defined media. This is significant as a large number of stem cells are required for many therapeutic applications and immunogenicity concerns abound with stem cell work [35,36]. Thus it is advantageous for a substrate platform to work in xenofree media.

In order to ensure clinical applicability, culture substrates should be compatible with common batch sterilization techniques

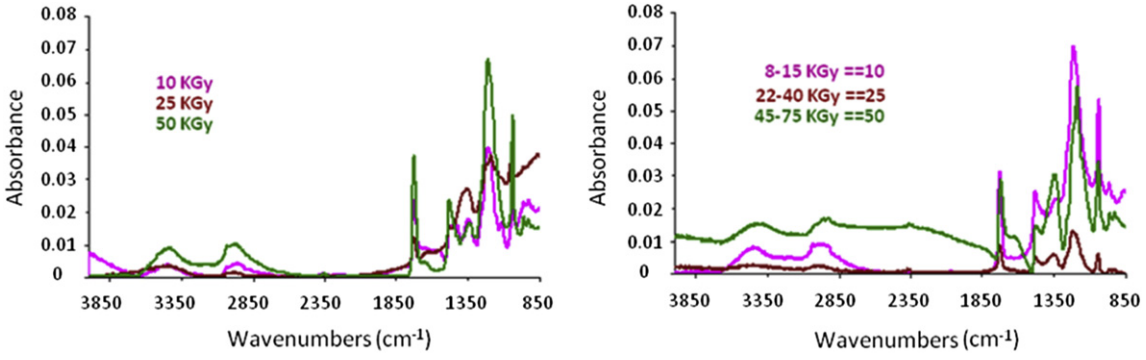


Fig. 5. FTIR of PMEDSAH coatings after exposure to various levels of e-beam- (left) and gamma- (right) radiation.

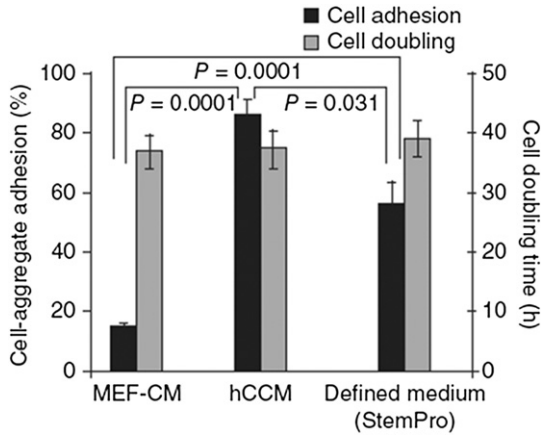


Fig. 6. PMEDSAH culture efficacy of hESCs in various media. Percentage (mean ± s.e.m.) of cell aggregate adhesion (number of aggregates attached with respect to total aggregates passaged) and population doubling time (twofold increase in colony area) for H9 hESCs cultured on PMEDSAH in several culture conditions. *P*-values calculated using unpaired *t*-test. Reproduced from [22] with permission.

such as e-beam- and gamma-radiation. The data presented in the previous section indicates compatibility of these methods for PMEDSAH-coated substrates. However, radiation-based sterilization has not been demonstrated for some of its counterparts. In particular, sterilization data is not available for GKKQRFRHRNRKG, human recombinant laminin-511, and hit 9. Additionally, large-scale radiation sterilization is likely to degrade peptide and protein containing culture systems [37]. Reusability is also an issue for peptide-based systems, Synthemax™ and GKKQRFRHRNRKG, as well as the recombinant laminin-511 system since they cannot be reused because peptides/proteins are subject to degradation by metalloproteases secreted by the cultured cells [38,39]. Degradation will lead to increased costs for end users as more culture substrates will be needed for a given task. The inclusion of peptides and recombinant proteins in the technologies also increases system costs as the peptides and proteins alone may be cost prohibitive [40]. Currently, Synthemax™ is the only commercially available synthetic stem cell culture platform and at a cost of $75 per 6-well plate, pricing may hinder access. Several stem cell types have clinical applicability including hESCs, human induced pluripotent stem cells (hiPSCs), and human mesenchymal stem cells (hMSCs). Thus in order to enhance wide-spread applicability, it is desirable for a cell culture system to promote long-term, undifferentiated culture for numerous stem cell lines and types. To date, PMEDSAH, Synthemax™, GKKQRFRHRNRKG, recombinant laminin-511, hit 9, and PMVE-alt-MA have all demonstrated efficacy with multiple stem cell types or stem cell lines. Table 3 that follows provides a comparison of the synthetic substrates described in this work, relative to one another and to Matrigel™ the current gold standard for stem cell culture in defined media conditions.

It is apparent that many efforts have been focused on maintaining hPSCs in the undifferentiated state in long-term culture. However, ultimately these hPSCs will be directed to specific cell lineages for various applications in tissue engineering and regenerative medicine. Therefore, a number of important studies have attempted directed differentiation of hPSCs [41,42]. Recently, hMSCs derived from hESCs have been encapsulated into poly(-ethylene glycol)-based (PEG-based) hydrogels containing ECM proteins [43]. In this instance, stem cells were cultured for up to 6 weeks in media conditions designed to direct cells to chondrogenic or osteogenic lineages. Results indicated that the type of ECM protein contained within the hydrogel influenced the degree to which differentiation markers were expressed. Others have tuned the mechanical properties of materials to guide differentiation. For example, hESCs were cultured on poly(dimethylsiloxane) substrates with augmented stiffness to determine the influence of this material property on the differentiation of hESCs [44]. The authors found that stiffness impacted primary germ layer differentiation and terminal differentiation to an osteogenic lineage. Increasing substrate stiffness from 0.041 MPa to 2.7 MPa led to an upregulation of mesodermic markers in the early stages and to enhanced osteogenic differentiation in the terminal stages. Systematic studies on the role of stem cells and stem cell sourcing are essential in developing clinically relevant bone [45].

4. Summary and future outlook

The generation of synthetic substrates for sustained pluripotent stem cell culture in defined and xenofree media is a significant step in the use of hPSCs in therapeutics as well as basic research. At this juncture, polymeric and peptide-based materials have been utilized to maintain hPSCs in an undifferentiated state. However, the mechanism(s) of action that result in the success of these substrates for prolonged culture have not been fully elucidated. Future work will aim to illuminate the influence and interaction of material parameters such as surface chemistry, hydrophilicity/hydrophobicity, stiffness, etc on stem cell maintenance. Doing so

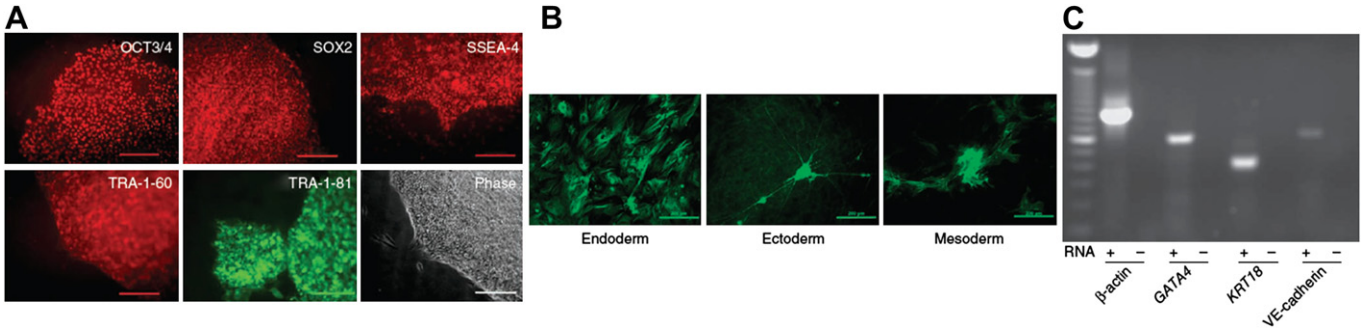


Fig. 7. Cellular characterization of hESCs cultured on PMEDSAH substrates in StemPro™ media. (A) Fluorescence micrographs of colonies of H9 cells cultured on PMEDSAH in StemPro medium showing expression of hESC markers and a phase-contrast image. Scale bars, 200 μm. (B) Micrographs showing immunoreactivity for α-fetoprotein (endoderm), β-III tubulin (ectoderm) and smooth muscle actin (mesoderm) indicating the pluripotent state of H9 cells cultured on PMEDSAH in StemPro™ medium. Scale bars, 200 μm. (C) RT-PCR analysis of RNA from embryoid bodies showing expression of endoderm (*GATA4*), ectoderm (*KRT18*) and mesoderm derivatives (*VE-cadherin*; also known as *CDH5*). Reproduced from [22] with permission.

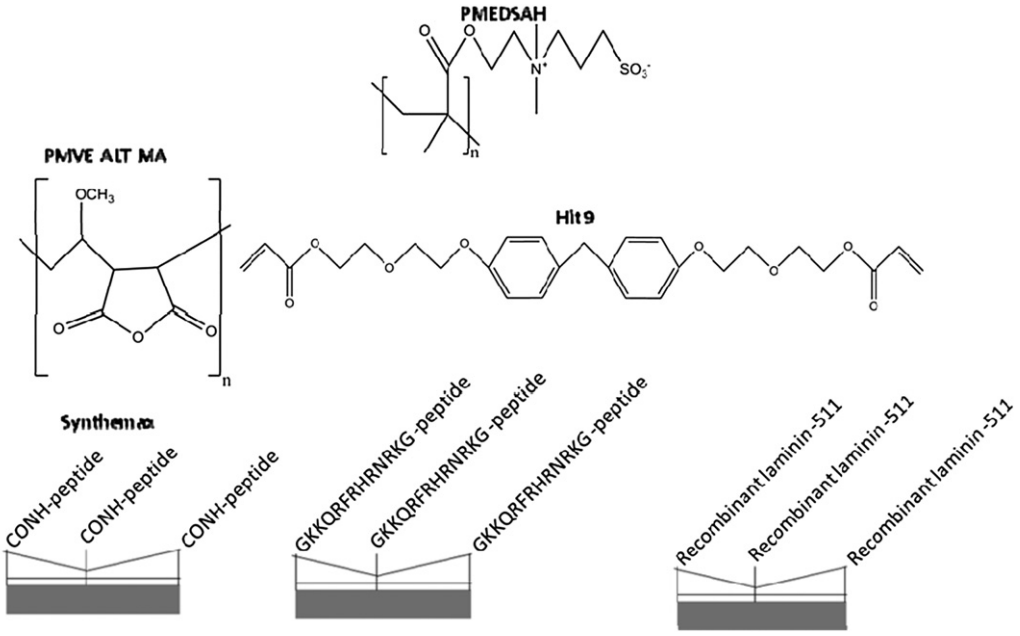


Fig. 8. Synthetic stem cell culture materials used for long-term maintenance of hPSCs.

Table 3
Comparison of synthetic substrates and Matrigel™ for long-term hPSC culture.

Substrate type	Reusable	Passage # tested	Prep for cell culture use	Can be sterilized via large batch methods?	Relative cost	Fabrication	Cell type
PMEDSAH	Yes	25	Used as is	Yes. E-beam- and gamma-radiation	Inexpensive	Polymeric grafting	hESC (several types)
Synthemax™ (Corning)	No (b/c peptide)	≥10	Used as is	Gamma radiation. Subject to degradation after gamma exposure (b/c peptide)	Expensive (b/c of peptides)	Photopolymerization & chemical conjugation of peptide via EDC/NHS	hESC (several types)
GKKQRFRHRNRKG	No (b/c peptide)	17	Used as is	Subject to degradation after gamma exposure (b/c peptide)	Expensive (b/c of peptides)	Physisorption	hiPSC and hESC
PMVE-alt-MA	Yes	5	Used as is	UVC germicidal radiation	Inexpensive	Free radical polymerization	hiPSC and hESC
hit 9	No (b/c of need of protein adsorption)	≥5	Yes. Requires preadsorption of ECM protein vitronectin.	Unknown	Inexpensive	Photopolymerization	hiPSC and hESC
Human recombinant laminin-511	No (b/c protein)	≥20	Used as is	Subject to degradation after gamma exposure (b/c peptide)	Expensive	Physisorption	hiPSC and hESC
Matrigel™	No	≥20	Yes	No	Expensive	Cell feeder layers	hiPSC, hESC, hMSCs, etc

will enhance the design of biomaterials for stem cell applications. Recently, a majority of research has focused on hESCs, however an optimal substrate would be compatible with multiple cell types and therefore future work will assess the efficacy of these substrates for the long-term culture of numerous cell types. Efficacy and immunogenicity are not the only considerations as cost also impacts technology adoption. Going forward, cost effective materials and processes such as those used to generate our groups' zwitterionic hydrogel will need to be explored.

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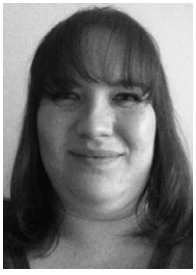

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