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Engineered Polymer-Media Interfaces for the Long-term Self-renewal of Human Embryonic Stem Cells

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Abstract

We have developed a synthetic polymer interface for the long-term self-renewal of human embryonic stem cells (hESCs) in defined media. We successfully cultured hESCs on hydrogel interfaces of aminopropylmethacrylamide (APMAAm) for over 20 passages in chemically-defined mTeSR^{TM} 1 media and demonstrated pluripotency of multiple hESC lines with immunostaining and quantitative RT-PCR studies. Results for hESC proliferation and pluripotency markers were both qualitatively and quantitatively similar to cells cultured on Matrigel^{TM}-coated substrates. Mechanistically, it was resolved that bovine serum albumin (BSA) in the mTeSR^{TM} 1 media was critical for cell adhesion on APMAAm hydrogel interfaces. This study uniquely identified a robust long-term culture surface for the self-renewal of hESCs without the use of biologic coatings (e.g., peptides, proteins, or Matrigel^{TM}) in completely chemically-defined media that employed practical culturing techniques amenable to clinical-scale cell expansion.

Keywords

Biointerface; Hydrogel; Human Embryonic Stem Cell; Self-Renewal; BSA; QCM-D

1. Introduction

Human embryonic stem cells (hESCs) are being studied as a potential source of cells for the treatment of many diseases (e.g. cardiac repair, diabetes, spinal cord injury, Parkinson's, leukemia, etc.). These same cells are also being touted as an ideal cell source for *ex vivo* tissue engineering for drug screening or *in situ* regenerative medicine. The successful integration of hESCs into such approaches will require large-scale cell expansion without differentiation (i.e., self-renewal). However, it is difficult to precisely control the behavior of hESCs *in vitro*, since environmental conditions for self-renewal and differentiation are neither completely understood nor defined.

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Originally, hESCs were grown in monolayer culture with a feeder layer of mouse cells or with conditioned media derived from these feeder cells. Rapid advancement in cell culture techniques has led to the development of chemically-defined media and feeder-free hESC culture systems that employ animal or human-derived extracellular matrix (ECM) proteins to coat the culture substrata.[1–10] For chemically-defined media, Matrigel™ is the most prevalent ECM analogue, [11] which is an extraction from Engelbreth–Holm–Swarm (EHS) mouse sarcomas that contains not only basement membrane components (laminin, collagen IV, heparin sulfate proteoglycans and entactin), but also matrix degrading enzymes, their inhibitors, numerous growth factors, and a broad variety of other proteins, as recent proteomic data indicate.[12] As such, Matrigel™ represents a poorly defined substrate for precise hESC expansion. Therefore, more recent advances have focused on replacing Matrigel™ and isolated ECM proteins with recombinant proteins, [13] synthetic peptides, [5, 14–20] and/or polymers.[21, 22] Synthetic peptide interfaces have achieved the most promising results, having demonstrated short term and long-term (i.e., greater than 10 passages) expansion in chemically-defined media. However, there is still significant marginal cost associated with these systems, potentially limiting their use in large-scale manufacturing, where a requirement to deliver 10^9 cells per patient is estimated for many stem cell therapies and diagnostic screenings. A completely defined hESC culture system exploiting a synthetic polymer interface would minimize cost, bring the practice in line with current culture methods, and aid in standardizing the process. To date, synthetic surfaces developed for hESC culture have not achieved consistent long-term self-renewal of hESCs in chemically-defined media.[21, 22]

2. Materials and Methods

2.1 Network Polymerization on TCPS

Costar (Corning; Corning, NY) 12-well tissue culture polystyrene (TCPS) plates were used for all cell culture experiments. 12-well plates were activated by a Plasma Preen II 973 Oxygen Plasma (Plasmatic Systems) set at 1 Torr and 150 W for 1 min. Subsequently, hydrogel network coatings were polymerized directly onto the bottom of each well of a 12-well plate via photoinitiated radical addition polymerization. 200 μ L of a solution containing monomer, crosslinker, and photoinitiator was pipetted into each well: 0.15 g/mL N-(3-Aminopropyl)methacrylamide hydrochloride (APMAAm; Polysciences, Warrington, PA), 0.0015 g/mL N,N-methylenebis(acrylamide) (BIS, Polysciences), and 0.005 g/mL Irgacure 2959 (Ciba) in 97:3 (v/v) water:isopropanol (IPA; Sigma Aldrich). The samples underwent photoinitiated polymerization for 1 min using a UV light source (UV light irradiation of 0.36 mW/cm² at 365 nm). Excess solution was aspirated from wells and then wells were rinsed 3 times with water to remove unreacted materials. Plates were sterilized by soaking in 70:30 (v/v) ethanol:water mix for 20 min followed by 3 rinses in DPBS. All water used in this study was ultra pure ASTM Type I reagent grade water (18 M Ω · cm, pyrogenfree, endotoxin <0.03 EU/ml).

2.2 Network Polymerization on QCM-D crystals and Si wafers

QCM-D sensor crystals (QSX101, Qsense) and Si-wafer pieces (1 cm × 1 cm) were cleaned by soaking in water, acetone, and toluene. Polystyrene (PS) films were spin-coated onto QCM-D sensor crystals and Si-wafers at 2000 rpm for 60 secs from a 1% (w/v) PS solution in toluene as described previously.[23] PS films were annealed for 48 hours at 110°C and subsequently activated by oxygen plasma (Plasmatic Systems) set at 1 Torr and 150 W for 1 min. Hydrogel coatings were photo-polymerized directly onto the PS layer by flipping the samples upside down in a 6-well PS plate with 500 μ L of monomer solution per well. The solution was identical to above aside from the solvent, which was 100% water. The samples underwent photo-initiated polymerization for 1 min using a UV light source (identical

parameters as listed in Section 2.1) and were rinsed 3x in water. In addition, the presence of the APMAAm surface was validated by XPS measurements on Si-wafer samples (SI Figure 1) showing an increase in the N peak as shown in the TCPS-APMAAm samples.

2.3 X-ray Photoelectron Spectroscopy (XPS)

APMAAm-modified 12-well TCPS plates were analyzed with XPS where all spectra were taken on a Surface Science Instruments S-probe spectrometer with a monochromatized Al K α X-ray, and a low energy electron flood gun for charge neutralization of non-conducting samples. The samples were floated on double sided tape and run as insulators. Three spots were analyzed on each sample. Samples were analyzed with a pass energy of 150 eV for survey spectra and 50 eV for high resolution scans, and a take-off angle of 55°. Service Physics ESCA2000A Analysis Software was used for peak-fitting. The binding energy scale of the high-resolution spectra was calibrated by setting the C 1s to 285.0 eV.

2.4 Contact Angle Goniometry

Water contact angles of as received TCPS and APMAAm substrates (quasi-static advancing ($\theta_{ADV}^{H_2O}$)) were measured according to methods previously described[24] using a customized micrometer microscope fitted with a goniometer eyepiece (Gaertner, Chicago, IL). All contact angles were measured at ambient temperature to the nearest degree.

2.5 hES cell cultures

H1s[25] and H9-hOct4-pGZ (hOct4 promoter driving GFP and Zeo) from Wicell were employed in this work. hESCs were cultured on APMAAm gels and Matrigel™ controls in chemically defined mTeSR™ 1 media (Stem Cell Technologies, Vancouver, BC). Cells were fed daily and passaged 1:3–1:6 every 3–5 days by exposure to Collagenase IV (Gibco Invitrogen; Carlsbad, Ca) at 200 U/mL in Knockout DMEM (KO-DMEM; Gibco Invitrogen) for 5 min at room temperature (RT). Cells were then washed on the dish with Dulbecco's Phosphate Buffered Saline (DPBS; Gibco Invitrogen), followed by mTeSR™ 1 media containing 5 μ M Rock Inhibitor (Ri; Calbiochem EMD Chemicals). Cells were gently scraped and pipetted into smaller colonies, and passaged in mTeSR™ 1 media supplemented with Ri. For controls, Matrigel™ was diluted 1:30 in KO-DMEM at 4°C, allowed to adsorb for more than 10 min at RT, and then aspirated immediately before use.

2.6 hES cell differentiation

H1s and H9-hOct4-pGZ were differentiated by normal passaging and suspension in 20% FBS in KO DMEM (Gibco Invitrogen). At Day 8, EBs were plated on gelatin-coated TCPS wells and immunostaining experiments were carried out at day 40.

2.7 Karyotype

After 10 passages on APMAAm, hESCs were passaged back onto Matrigel™ and brought to Children's Hospital Oakland Cytogenetics laboratory for karyotyping by GTG-banding.

2.8 Immunostaining and Quantitative Analysis

Samples in 12-well plates were fixed using 4% (v/v) paraformaldehyde in DPBS at 37°C for 10 min. Samples were then rinsed 3x in PBS and kept at 4°C. Cells were permeabilized with 0.1% Triton-X (Sigma) for 10 min; for intracellular markers, cells were further permeabilized with 0.5% SDS for 5 min. Cells were incubated with a 1:100 dilution of primary antibody [Mouse Anti-Oct-4 IgG (Santa Cruz Biotechnology); Mouse Anti-SSEA-4 IgG (Millipore); Mouse Anti-Tra-1-60 IgM (Millipore); Rabbit Anti-Desmin IgG (Thermo Scientific); Rabbit Anti- α -Smooth Muscle Actin IgG (Millipore); Mouse Anti-Human B

Tubulin III IgG (Millipore)] overnight at 4°C. The next day, cells were incubated with an appropriate Alexa Fluor secondary antibody 1:300 for 1 hr at RT [Goat Anti-Mouse AlexaFluor 488 IgG (Molecular Probes), Goat Anti-Mouse AlexaFluor 488 IgM (Molecular Probes), Goat Anti-Rabbit AlexaFluor 546 IgG (Molecular Probes)]. Finally, cell nuclei were stained with 4',6-diamino-2-diamidino-2-phenylindole, diacetate (DAPI; Molecular Probes) for 5 min at RT. All staining steps were followed by 3 washes in PBS. Cells were visualized immediately as follows: Epifluorescent imaging (Axiovert), whole plate imaging (ImageXpressMicroscope), and Confocal imaging (Zeiss). For quantitative image analysis, the Metamorph software was used and the 'Cell scoring' algorithm was applied. Briefly, the cell nuclei were identified by DAPI staining and the corresponding area positive for a specific wavelength (i.e. AlexaFluor 488, AlexaFluor 546) was measured. The percent of cells that were associated with a positive stain was quantified for 49 different sites (1000 $\mu\text{m}^2/\text{site}$) on each well. Data is presented as the mean percentage of positive cells on all sites $\pm$ SEM.

2.9 Quantitative RT-PCR

RNA and cDNA were attained from cells using TaqMan Fast Cells-to-CT Kit (Applied Biosystems) according to the manufacturer's instructions for n=3 biological replicates. cDNA and RT-PCR reactions were performed with a StepOnePlus instrument (Applied Biosystems) using TaqMan Fast Universal PCR Master Mix, and TaqMan Gene Expression Assays (GAPDH HS9999905_m1; Oct4 Hs00742896_s1; Nanog Hs02387400_g1; Sox2 Hs00602736_s1) according to the manufacturer's instructions.

2.10 Quartz Crystal Microbalance with Dissipation (QCM-D) experiments

In a QCM-D, an AC voltage is pulsed across an AT-cut piezoelectric quartz crystal, causing it to oscillate in shear mode at its resonant frequency. The resonant frequency of the crystal is recorded in real time and depends on the total oscillating mass and the intrinsic properties of the quartz crystal. The Sauerbrey relationship[26] states that a change in the mass (ΔM) of a film or adlayer is directly proportional to a change in the normalized resonant frequency of the crystal (ΔF):

$$\Delta M = -C * \Delta F/n$$

where C is the mass sensitivity constant of $-17.7 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ and n is the overtone number. The ΔF is due to the change in total coupled mass, including hydrodynamically coupled water and water associated with adsorbed molecules. The dampening of the shear wave is also recorded simultaneously with the resonant frequency of the crystal as the dissipation factor (D), which is the ratio of the dissipated energy to the stored energy. For this work, APMAAm-modified sensor crystals were loaded into the QCM-D (E4, Biolin Scientific, Sweden) and solutions were flowed over the surface of the crystal at 400 $\mu\text{L}/\text{min}$ using a Peristaltic pump (Ismatec IPC-N4; Glattbrugg, Switzerland). Frequency (F) and dissipation were recorded in real time at 4 different overtones: n=1, 3, 5, and 7. Initially, DPBS flowed over the APMAAm surface to establish a baseline for the F and D values of the crystal. Once F and D were stabilized, different solutions were introduced sequentially until the measurement reached equilibrium. All calculations were done with n=7 overtone since it contained the least amount of noise.

2.11 Cell Attachment Studies

hESCs were passaged normally and plated in the specified media. After 24 h, hESCs were washed in PBS and frozen at -20°C immediately. Cyquant (Molecular Probes) was used to quantify cell attachment according to the manufacturer's instructions. Briefly, cells were

simultaneously lysed and incubated with a proprietary green fluorescent dye, which fluoresces when bound to nucleic acids for 5 min. After the incubation time, solution was pipetted onto a 96-well black plate (Costar) and the fluorescence was measured employing a Spectramax GeminiXS spectrofluorometer (Molecular Devices, CA; ex/em/cutoff, 480/520/515 nm). The population doubling time (PDT) was calculated according to the following equation:[27]

$$PDT=(T_2 - T_1)/[3.32*(\log N_2 - \log N_1)],$$

Where T_1 was day 1, T_2 was day 4, N_1 was the number of cells at T_1 , and N_2 was the number of cells at T_2 .

2.12 Protein Spreading Experiments

QCM-D sensor crystals were modified as described above with spin-coated PS, followed by synthesis of APMAAm films. First, F and D measurements of the modified crystals were allowed to stabilize in PBS in the QCM-D. Next, one of four BSA solutions in PBS: 0.01 mg/mL BSA, 0.025 mg/mL BSA, 0.05 mg/mL BSA, and 0.1 mg/mL BSA was introduced into each chamber. Solutions were flowed over the surface of the crystal at 200 μ L/min until the F and D signals were equilibrated. Finally samples were rinsed with PBS. The Sauerbrey relationship was used with F7/7 measurements to calculate the total mass of the film adsorbed. The τ -75 was defined as the time for each experiment to reach 75% of the total saturated mass adsorbed. From this total mass, the footprint size was calculated.

2.13 Statistics

All data were expressed as the average of at least three replicate experiments $\pm$ the standard error of the mean. Statistical comparisons were performed by ANOVA ($P < 0.05$) followed by Holms t -tests ($P < 0.05$) for significance.

3. Results

3.1 Design and characterization of APMAAm surfaces

We have developed a synthetic polymer interface for the long-term self-renewal of hESCs. The hydrogel network coating was comprised of aminopropylmethacrylamide (APMAAm) monomer and *N,N*-methylenebis(acrylamide) (bis) crosslinker that was grafted to standard tissue culture polystyrene (TCPS) dishes via photoinitiated addition polymerization. We verified the polymerization reaction with X-ray photoelectron spectroscopy (XPS) (Fig 1a–d; Table 1; Table 2) and contact angle goniometry. The photoemission data consistently showed increased N and corresponding decreased C on the APMAAm samples compared to the as received TCPS control. After polymerization, the advancing water contact angles ($\theta_{ADV}^{H_2O}$) changed from $72.6 \pm 0.3^\circ$ to $35.3 \pm 0.3^\circ$, in agreement with previously published data for self-assembled monolayer (SAM) alkanethiolates and organosilanes presenting a terminal amine.[28, 29]

3.2 Long-term culture of multiple hESC lines on APMAAm surfaces

The APMAAm networks maintained hESC pluripotency for long-term culture. We cultured both H1s and H9-hOct4-pGZ cell lines on APMAAm substrates for 10 passages (p10) in chemically-defined mTeSR^{TM} 1 media, and characterized the pluripotency of both cell lines compared to Matrigel^{TM}-coated substrata. Throughout 10 passages, H1s and H9-hOct4-pGZs maintained typical stem cell morphology and grew in colonies similar to Matrigel^{TM} controls

(Fig. 2a,b). The pluripotency of both lines was confirmed via immunostaining, where the expression levels of pluripotency markers of H1s and H9-hOct4-pGZs were similar to Matrigel™ controls (Fig. 2c–h). Quantitative analysis of pluripotency markers (Fig. 2m) and gene expression (Fig. 2o) indicated that the APMAAm networks were robust in maintaining pluripotency for H1s similar to Matrigel™. For the H9-hOct4-pGZs, APMAAm interfaces maintained pluripotent markers to a greater degree than Matrigel™ (Fig. 2n). Due to the genetic modifications made to the H9 line, these cells spontaneously differentiate at high passages in culture. Finally, the karyotype of both cell lines was normal after culture on APMAAm networks (Fig. 2j shows H1 karyotype).

3.3 hESC attachment and proliferation

Interfaces of APMAAm networks were as effective as Matrigel™-coated substrata in supporting the proliferation of hESCs. H1s and H9-hOct4-pGZs were cultured on APMAAm substrates in mTeSR™ 1 media and compared to Matrigel™. On the first passage from Matrigel™, H9-hOct4-pGZ attachment on APMAAm was approximately half of that on Matrigel™ (Figure 2i). However, the hESCs adapted to the APMAAm substrate, where the number of cells attached to APMAAm increased to $63.3 \pm 0.04\%$ relative to adhesion on Matrigel™ at p22 (Figure 2i). Although there were initially fewer cells attached on APMAAm, the proliferation of the H9-hOct4-pGZs was faster than Matrigel™ at both passages 1 and 22 (Figure 2k & l). The population doubling time of the H9-hOct4-pGZs at passage 1 on APMAAm was 22.4 h, compared with 26.1 h on Matrigel™. At passage 22 on the APMAAm (total passage number of 80), the H9-hOct4-pGZs slowed their proliferation to a population doubling time of 54.0 h, compared with 88.8 h on Matrigel™.

3.3 hESC differentiation and germ layer formation

hESCs cultured on APMAAm interfaces were differentiated into embryoid bodies (EBs) to demonstrate formation into all three germ layers. H9-hOct4-pGZs cultured on APMAAm for 12 passages formed nearly spherical EBs with typical morphology (Fig. 3a). At Day 40 after EB formation, immunostaining results demonstrated the formation of all three germ layers in EBs on Matrigel™ and APMAAm samples (Figure 3c–h) indicating that the cells retain the multilineage potential after culture on APMAAm surfaces.

3.4 Mechanism of hESC attachment

As the interface was not functionalized with peptides or proteins to promote cell adhesion, we sought to understand the mechanism for hESC attachment to the APMAAm networks. We employed a QCM-D to record molecular adsorption from the mTeSR™ 1 media to the APMAAm interface in real time. When the mTeSR™ 1 media was introduced to the QCM-D chamber, there was a decrease in the frequency of the crystal (ΔF ; Fig 4a) and an increase in the dissipation factor (ΔD ; Fig 4b). As the ΔD was low and the ΔF curves of the different overtones were nearly overlapping, we treated the adsorbed film as a rigid elastic film. According to the Sauerbrey relationship, the observed decrease in F was equivalent to the adsorption of a layer of $\sim 620 \text{ ng/cm}^2$ within 15 minutes of exposure (Fig. 4a). This mass includes any coupled water, which can contribute significantly to the mass of the adsorbed layer.[30] Next, approximately 26% of the layer was desorbed when DPBS was introduced into the chamber following the mTeSR™ 1 media, resulting in a final film mass of $\sim 460 \text{ ng/cm}^2$. Due to the significant mass of the adsorbed film and the classic Langmuir adsorption isotherm obtained, we hypothesized that this layer was comprised of proteins from the mTeSR™ 1 media.

With evidence that a macromolecular layer was adsorbing to the APMAAm interface, we sought to understand which molecule in this layer promoted hESC attachment. The complete mTeSR™ 1 media contained the following 3 proteins that we hypothesized may have played

a role; bovine serum albumin (BSA; [12.9 mg/mL]), transforming growth factor beta (TGF- β ; [1 ng/mL]), and basic fibroblastic growth factor (bFGF; [0.1 μ g/mL]). We explored the role of these proteins for H9-hOct4-pGZ attachment to APMAAm by performing cell attachment studies with imTeSR^{TM} 1 (incomplete; basal media without the frozen supplement containing the proteins) supplemented with the aforementioned individual proteins at levels in the complete media. The number of cells attached after 24 hours in different media conditions on APMAAm is shown in Figure 5a. The addition of BSA to imTeSR^{TM} 1 resulted in four times the number of hESCs attached compared to the imTeSR^{TM} 1 with either bFGF or TGF- β . Furthermore, imTeSR^{TM} 1 with BSA alone led to nearly twice the hESC attachment compared to the complete mTeSR^{TM} 1 media. These observations indicated that BSA in the mTeSR^{TM} 1 was the principal protein responsible for hESC attachment and that other molecules in the complete media might be competing with BSA for adsorption sites on the APMAAm network, reducing hESC adhesion to the surface.

We performed additional protein adsorption experiments with the QCM-D to verify adsorption of BSA onto the APMAAm surface. In these experiments, we employed the incomplete media similarly to the cell attachment experiments. First, imTeSR^{TM} 1 was introduced into the chamber, resulting in the adsorption of ~ 10 ng/cm² layer (Fig 4c). This layer was likely comprised of amino acids and lipids present in the basal media. When the imTeSR^{TM} 1 with BSA was sequentially introduced into the chamber, a layer of ~ 1080 ng/cm² of BSA adsorbed onto the APMAAm within 15 min, a greater mass than from the complete media. Assuming a maximum-packed layer of end-on adsorbed BSA (~ 14 nm $\times$ 4 nm $\times$ 4 nm), a monolayer of BSA corresponds to a mass of ~ 790 ng/cm²; however this does not include the mass of coupled water. The change in dissipation (ΔD) was 7.5×10^{-6} (Fig 4d), indicating that the protein layer was less rigid than the film adsorbed from the complete media (ΔD of 5.0×10^{-6}). After the preadsorption of the BSA, very little desorption occurred when complete mTeSR^{TM} 1 media was sequentially introduced into the chamber. However, when DPBS was introduced into the chamber, 25% of the film was desorbed. The final surface density of the film was ~ 810 ng/cm², compared to ~ 460 ng/cm² of protein from the complete media.

In order to further analyze the BSA layer adsorbed to the APMAAm surface, we conducted BSA spreading experiments as described elsewhere [31, 32]. A QCM-D was employed to record the adsorption of BSA from solutions at different concentrations in PBS onto APMAAm surfaces. Based on the results from these experiments, the footprint size of BSA molecules was determined at τ -75, defined as the time at which 75% of the surface saturation was reached. At different solution concentrations of BSA, the adsorption resulted in different footprint values. As the BSA solution became more dilute, the footprint size increased (Figure 5b), indicating molecular spreading on the APMAAm surfaces. However, when BSA was adsorbed from incomplete mTeSR^{TM} 1 media the adsorption resulted in the smallest footprint size, approaching the theoretical end-on adsorption dimension of ~ 16 nm², and no spreading of the molecule on the surface. As shown in Figure 5a, the incomplete mTeSR^{TM} 1 media supplemented with BSA resulted in the highest number of cells attached, which indicated the APMAAm surface adsorbs BSA in an unfolded state, allowing for hESC attachment, growth, and self-renewal for extensive passages.

4. Discussion

For the clinical application of hESCs there is a critical need for cell culture systems with defined environments that precisely control the behavior of hESCs *in vitro*. Much of the research in this field previously [15–22] has employed preadsorption of proteins or peptide to surfaces which increase cost and limit scalability, or have failed to truly demonstrate long-term hESC culture. In this work, we have created a unique polymer interface for the long-

term self-renewal of hESCs. We believe this same system has the potential to be used for both self-renewal of human induced pluripotent stem (hiPS) cells, and directed differentiation of hESCs and hiPS into specific lineages under the appropriate, defined media conditions.

We identified that BSA adsorbing from the mTeSR^{TM}1 media played a key role in hESC attachment to APMAAm interfaces. BSA is a large serum protein (MW = 66 kDa) traditionally used in immunoassays as a blocking protein due to its stability and lack of involvement in most biochemical reactions.[33] However, in our work the specific conformation of the BSA on our aminated interface was crucial for enhanced hESC attachment and potentially led to a facile interface that supported long term growth (over 20 passages) of hESC lines. The adsorption of BSA from imTeSR^{TM}1 resulted in maximal adsorbed mass, with a theoretical end-on adsorption dimension of $\sim 16 \text{ nm}^2$. This interface also supported the maximal amount of cell adhesion. Cell adhesion to interfaces created from either bFGF or TGF- β adsorbed from imTeSR^{TM}1 was lower than that from complete mTeSR^{TM}1 and significantly lower than BSA adsorbed from imTeSR^{TM}1. The lower mass of protein adsorbed to the APMAAm interfaces from the complete media indicated competition from smaller molecules occupying adsorption sites on the surface. Collectively, these data suggest that the protein layer adsorbing to the APMAAm was primarily BSA, although this does not preclude the involvement of other molecules in the media playing a role in hESC attachment. Amino acids, lipids, vitamins, hormones and other molecules could be co-adsorbing with the BSA and affecting hESC adhesion; however the BSA was clearly a necessary component in hESC attachment.

Cell attachment mediated by BSA adsorption has been observed previously on aminated surfaces. Bekos, et al., [34] and Ranieri, et al., [35] reported that BSA adsorbed to aminated polymer films of poly(tetrafluoroethylene-co-hexafluoropropylene) that had been treated with radiofrequency glow charge plasma (FEP-OH), and subsequently modified with an (aminopropyl)trimethoxysilane (resulting in an aminated surface) showed significantly increased mouse neuroblastoma cell (NB2a) and rat endothelial cell (REC) attachment compared with the hydroxylated FEP-OH surface. In addition, by attaching fluorescent markers that detected protein unfolding, they showed that unlike adsorption on hydroxyl surfaces, BSA unfolded on the aminated surfaces. In contrast, although BSA spreading was demonstrated on APMAAm surfaces at very low concentrations, the condition that allowed for the greatest hESC attachment occurred when the BSA did not change conformation upon adsorption.

5. Conclusions

We have developed a completely synthetic and defined culture system that allows for long-term hESC growth and self-renewal. The primary advantage of this system is it does not require the prior attachment of peptides or proteins to promote cell attachment, is scalable, low cost, and is free of complex, undefined culture conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ludwig TE, Bergendahl V, Levenstein ME, Yu JY, Probasco MD, Thomson JA. Feeder-independent culture of human embryonic stem cells. *Nat Methods*. 2006; 3(8):637–646. [PubMed: 16862139]
2. Mallon BS, Park KY, Chen KG, Hamilton RS, McKay RDG. Toward xeno-free culture of human embryonic stem cells. *Int J Biochem Cell Biol*. 2006; 38(7):1063–1075. [PubMed: 16469522]
3. Wang L, Schuiz TC, Sherrer ES, Dauphin DS, Shin S, Nelson AM, et al. Self-renewal of human embryonic stem cells requires insuhn-like growth factor-1 receptor and ERBB2 receptor signaling. *Blood*. 2007; 110:4111–4119. [PubMed: 17761519]
4. Xu CH, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, et al. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol*. 2001; 19(10):971–974. [PubMed: 11581665]
5. Li YJ, Chung EH, Rodriguez RT, Firpo MT, Healy KE. Hydrogels as artificial matrices for human embryonic stem cell self-renewal. *J Biomed Mater Res A*. 2006; 79A(1):1–5. [PubMed: 16741988]
6. Beattie GM, Lopez AD, Bucay N, Hinton A, Firpo MT, King CC, et al. Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells*. 2005; 23(4):489–495. [PubMed: 15790770]
7. Xiao L, Yuan X, Sharkis SJ. Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. *Stem Cells*. 2006; 24(6):1476–1486. [PubMed: 16456129]
8. Yao S, Chen S, Clark J, Hao E, Beattie GM, Hayek A, et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proc Natl Acad Sci U S A*. 2006; 103(18):6907–6912. [PubMed: 16632596]
9. Fletcher JM, Ferrier PM, Gardner JO, Harkness L, Dhanjal S, Serhal P, et al. Variations in humanized and defined culture conditions supporting derivation of new human embryonic stem cell lines. *Cloning and Stem Cells*. 2006; 8(4):319–334. [PubMed: 17196096]
10. Liu YX, Song ZH, Zhao Y, Qin H, Cai J, Zhang H, et al. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. *Biochem Biophys Res Commun*. 2006; 346(1):131–139. [PubMed: 16753134]
11. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type-IV procollagen, laminin, and heparan-sulfate proteoglycan from the EHS sarcoma. *Biochem*. 1982; 21(24):6188–6193. [PubMed: 6217835]
12. Hansen KC, Kiemele L, Maller O, O'Brien J, Shankar A, Fornetti J, et al. An in-solution ultrasonication-assisted digestion method for improved extracellular matrix proteome coverage. *Mol Cell Proteomics*. 2009; 8(7):1648–1657. [PubMed: 19351662]
13. Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, et al. Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol*. 2006; 24(2):185–187. [PubMed: 16388305]
14. Meng Y, Eshghi S, Li YJ, Schmidt R, Schaffer DV, Healy KE. Characterization of integrin engagement during defined human embryonic stem cell culture. *FASEB J*. 2010; 24(4):1056–1065. [PubMed: 19933311]
15. Derda R, Li LY, Orner BP, Lewis RL, Thomson JA, Kiessling LL. Defined substrates for human embryonic stem cell growth identified from surface arrays. *ACS Chem Biol*. 2007; 2(5):347–355. [PubMed: 17480050]
16. Derda R, Musah S, Orner BP, Klim JR, Li LY, Kiessling LL. High-Throughput Discovery of Synthetic Surfaces That Support Proliferation of Pluripotent Cells. *J Am Chem Soc*. 2010; 132(4):1289–1295. [PubMed: 20067240]
17. Mei Y, Saha K, Bogatyrev SR, Yang J, Hook AL, Kalcioğlu ZI, et al. Combinatorial development of biomaterials for clonal growth of human pluripotent stem cells. *Nat Mater*. 2010; 9(9):768–778. [PubMed: 20729850]
18. Melkounian Z, Weber JL, Weber DM, Fadeev AG, Zhou YE, Dolley-Sonneville P, et al. Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nat Biotechnol*. 2010; 28(6):606–U695. [PubMed: 20512120]

19. Klim RK, Li L, Wrighton PJ, Piekarczyk MS, Kiessling LL. A Defined Glycosaminoglycan-binding Substratum for Human Pluripotent Stem Cells. *Nat Methods*. 2010; 7(12):989–994. [PubMed: 21076418]
20. Kolhar P, Kotamraju VR, Hikita ST, Clegg DO, Ruoslahti E. Synthetic surfaces for human embryonic stem cell culture. *J Biotechnol*. 2010; 146(3):143–146. [PubMed: 20132848]
21. Villa-Diaz LG, Nandivada H, Ding J, Nogueira-De-Souza NC, Krebsbach PH, O'Shea KS, et al. Synthetic polymer coatings for long-term growth of human embryonic stem cells. *Nat Biotechnol*. 2010; 28(6):581–583. [PubMed: 20512122]
22. Brafman DA, Chang CW, Fernandez A, Willert K, Varghese S, Chien S. Long-term human pluripotent stem cell self-renewal on synthetic polymer surfaces. *Biomaterials*. 2010; 31(34):9135–9144. [PubMed: 20817292]
23. Kohen NT, Little LE, Healy KE. Characterization of Matrigel interfaces during defined human embryonic stem cell culture. *Biointerphases*. 2009; 4(4):69–79. [PubMed: 20408727]
24. Bearinger JP, Castner DG, Golledge SL, Rezanian A, Hubchak S, Healy KE. P(AAm-co-EG) interpenetrating polymer networks grafted to oxide surfaces: Surface characterization, protein adsorption, and cell detachment studies. *Langmuir*. 1997; 13:5175–5183.
25. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998; 282(5391):1145–1147. [PubMed: 9804556]
26. Sauerbrey G. Verwendung van Schwingquarzen zur Wagung diinner Schichten und zur Mikrowagung. *Z Phys*. 1959; 155(206)
27. Davis, J. *Basic Cell Culture: a Practical Approach*. New York: Oxford; 1994.
28. Healy KE, Thomas CH, Rezanian A, Kim JE, McKeown PJ, Lom B, et al. Kinetics of bone cell organization and mineralization on materials with patterned surface chemistry. *Biomaterials*. 1996; 17(2):195–208. [PubMed: 8624396]
29. Stenger DA, Georger JH, Dulcey CS, Hickman JJ, Rudolph AS, Nielsen TB, et al. Coplanar molecular assemblies of aminoalkylsilane and perfluorinated alkylsilane - characterization and geometric definition of mammalian cell adhesion and growth. *J Am Chem Society*. 1992; 114(22):8435–8442.
30. Hook F, Kasemo B, Nylander T, Fant C, Sott K, Elwing H. Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during adsorption and cross-linking: A quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study. *Analytical Chem*. 2001; 73(24):5796–5804.
31. Wertz CF, Santore MM. Effect of surface hydrophobicity on adsorption and relaxation kinetics of albumin and fibrinogen: Single-species and competitive behavior. *Langmuir*. 2001; 17(10):3006–3016.
32. Santore MM, Wertz CF. Protein spreading kinetics at liquid-solid interfaces via an adsorption probe method. *Langmuir*. 2005; 21(22):10172–10178. [PubMed: 16229542]
33. Smith RA, Mosesson MW, Daniels AU, Gartner TK. Adhesion of microvascular endothelial cells to metallic implant surfaces. *J Mater Sci Mater Med*. 2000; 11(5):279–285. [PubMed: 15348024]
34. Bekos EJ, Ranieri JP, Aebischer P, Gardella JA, Bright FV. Structural changes of bovine serum-albumin upon adsorption to modified fluoropolymer substrates used for neural cell attachment studies. *Langmuir*. 1995; 11(3):984–989.
35. Ranieri JP, Bellamkonda R, Jacob J, Vargo TG, Gardella JA, Aebischer P. Selective neuronal cell attachment to a covalently patterned monoamine on fluorinated ethylene-propylene films. *J Biomed Mater Res*. 1993; 27(7):917–925. [PubMed: 8360219]

Figure 1. Hydrogel surfaces of aminopropylmethacrylamide (APMAAm) were synthesized and characterized

(a) Survey data from XPS analysis of APMAAm-modified TCPS. (b) Survey data from XPS analysis of TCPS.

Figure 2. Pluripotency of H1s and H9-hOct4-pGZs was maintained for 10 passages on APMAAm

Representative phase contrast images of H1s on (a) APMAAm and (b) Matrigel^{TM}-coated substrates at p8 (scale bar = 25 μ m). Representative confocal image stacks at p10 of H1 colonies on APMAAm stained with (c) Dapi, (d) Oct-4, and (e) SSEA-4; compared with H1 colonies on Matrigel^{TM} stained with (f) Dapi, (g) Oct-4, (h) SSEA-4 (scale bar = 10 μ m). (i) H9-hOct4-pGZ cell attachment at p1 and p22 onto APMAAm and Matrigel^{TM} normalized to Matrigel^{TM}. (j) H1s and H9-hOct4-pGZs had a normal karyotype after p10 on APMAAm surfaces (H1 karyotype shown). (k) H9-hOct4-pGZ proliferation at p1 on APMAAm and Matrigel^{TM} surfaces (normalized to Matrigel^{TM} d1). (l) H9-hOct4-pGZ proliferation at p22 on APMAAm and Matrigel^{TM} (normalized to Matrigel^{TM} d1). Quantitative immunostaining of (m) H1s and (n) H9-hOct4-pGZ for Oct4, SSEA-4, and TRA-1-60 after p10 on APMAAm and Matrigel^{TM}. (o) Quantitative RT-PCR results for puripotent markers Oct4, Sox2, and Nanog at p10 (H1).

Figure 3. H9-hOct4-pGZ cells were differentiated into EBs after 12 passages on APMAAm and Matrigel™

Phase contrast images of Day 11 EBs from cells originally cultured on (a) APMAAm and (b) Matrigel™ surfaces. Immunostaining for germ layer markers on day 40 for EBs derived from cells cultured on APMAAm surfaces: (c) Desmin (endoderm); (d) Smooth Muscle Actin (SMA; mesoderm); (e) β -Tubulin III (ectoderm); and, on Matrigel™: (f) Desmin; (g) SMA; and (h) β -Tubulin III (scale bars = 10 μ m).

Figure 4. Protein adsorption to APMAAm from mTeSR™1

Kinetics of protein adsorption from mTeSR™1 complete media onto the APMAAm as determined by QCM-D. Initially APMAAm-modified sensor crystals were baselined in DPBS, where no adsorption to the surface occurs. Next, complete mTeSR™1 media was introduced into the chamber and resulted in a decrease in (a) frequency (ΔF) of the crystal (corresponding to an increase in adsorbed mass) and an increase in the (b) dissipation factor (D). After rinsing, the final mass of the adsorbed film was 460 ng/cm². QCM-D measurements of the (c) ΔF and (d) ΔD with the adsorption of a layer of BSA onto the APMAAm from incomplete mTeSR™1 media supplemented with only BSA. Subsequent introduction of complete mTeSR™1, followed by PBS, leads to a stable adsorbed protein film of 810 ng/cm².

Figure 5. BSA adsorption to APMAAm surfaces

(a) H9-hOct4-pGZ attachment to APMAAm surfaces after 24 h in complete mTeSR^{TM}1 and incomplete mTeSR^{TM}1 supplemented with: BSA, TGF-β or bFGF, (normalized to complete mTeSR^{TM}1). The addition of BSA to imTeSR^{TM}1 led to more than four times the number of hESCs attached compared to the imTeSR^{TM}1 with either bFGF or TGF-β. (b) A QCM-D was employed to record the adsorption of BSA from solutions at different concentrations in PBS onto APMAAm surfaces. The footprint size of BSA molecules at τ -75 was calculated, where larger footprint sizes resulted from lower concentration solutions indicating spreading. When BSA was adsorbed from incomplete mTeSR^{TM}1 media, it adsorbed in the smallest footprint size of $\sim 16\text{nm}^2$, which indicated no spreading.

Table 1

Chemical Composition of the APMAAm surface by XPS					
	C 1s	O 1s	N 1s	Cl 2p	Si 2p
APMAAm	82.9 ± 1.0	13.8 ± 0.4	2.9 ± 0.4	0.3 ± 0.1	0.2 ± 0.3
PS control	84.7 ± 0.4	15.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 2

High Resolution C 1s XPS					
	C-C	C-O _n N	C=O	O-C=O	Shake-up
APMAAm	82.1 ± 3.2	11.4 ± 2.7	4.6 ± 0.7	1.9 ± 1.7	0.0 ± 0.0
PS control	76.2 ± 7.7	10.6 ± 1.6	8.6 ± 4.2	2.7 ± 2.1	1.8 ± 1.6