Thermoresponsive poly(vinyl methyl ether) (PVME) retained by 3-aminopropyltriethoxysilane (APTES) network

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ABSTRACT

Thermoresponsive polymers (TRP)s have been widely used for various applications from controlling membrane fouling in separation to cell/cell sheet harvesting in regenerative medicine. While poly (*N*-isopropylacrylamide) (pNIPAAm) is the most commonly used TRP, less expensive and easily processed poly (vinyl methyl ether) (PVME) also shows a hydrophilic to hydrophobic transition at 32-35 °C, near physiological conditions. In this study, we investigated the processing conditions for retaining a stable layer of PVME thin film on silica surfaces via entrapment in a 3-aminopropyltriethoxysilane (APTES) network. In addition, the thermoresponsive behaviors (TRB) of the retained PVME films were evaluated. Blend thin films of PVME:APTES with 90:10 and 50:50 mass ratios were spin-coated from their solutions in ethanol under ambient conditions and then annealed in a vacuum oven at 40, 60, 80, or 120 °C for one, two or three days. The annealed films were then thoroughly rinsed by room temperature water and then soaked in water for 3 days. Our results showed that annealing at a temperature of ≥40 °C was necessary for retaining a PVME film on the surface. A higher annealing temperature led to a greater film retention, probably due to the formation of a tighter APTES network. Regardless of processing conditions, all retained PVME films showed TRB, determined by water contact angles below and above the transition temperature of PVME. Additionally, particle attachment and protein adsorption on retained PVME films showed lower attachment or adsorption at room temperature as compared to that at 37 °C,

and a greater difference was observed for the 90:10 blend where more PVME was consisted. Furthermore, human mesenchymal stem cells attached and proliferated on the retained PVME surfaces at 37 °C and rapidly detached at room temperature. These results illustrated the potential applications of PVME surfaces as thermoresponsive supports for low-fouling applications and non-invasive cell harvesting.

Keywords: Surface immobilization of poly (vinyl methyl ether), hydrophobic-hydrophilic transition, thermo-reversible protein adsorption, human mesenchymal stem cells (hMSCs), non-invasive cell harvesting.

1. INTRODUCTION

Stimuli-responsive polymers (SRP)s respond drastically to small environmental changes such as temperature, pH, ionic strength or redox conditions.[1] Thermoresponsive polymers (TRP)s exhibit an abrupt volume phase transition by increasing temperature above their hydrophilic hydrophobic transition or lower critical solution temperature (LCST).[2] TRPs with their LCST close to biological temperature,[3] such as poly(*N*-isopropylacrylamide) (pNIPAAm) and poly(vinyl methy lether) (PVME), are attractive candidates for a wide range of applications including drug delivery, cell culture supports, protein chromatography, sensing devices, and regenerative medicine. [4–9]

One of the most important steps in cell culture is the recovery of undamaged functional cells from cell culture substrates. Recently, stem cell-based regenerative therapy has attracted increasing attention as the most promising therapeutic method for treating defects or injuries to tissues and organs.[10–13] Human mesenchymal stem cells (hMSC)s are one of the most preferred cells for therapeutic and regenerative applications, since they could be isolated from a variety of tissues such as bone marrow, adipose tissue, cord blood, dental tissues and they exhibit noticeable regenerative capacity to differentiate into multi-lineage cells.[10,14] Two common approaches for cell harvesting include using proteolytic enzymes (such as trypsin, pronase, and collagenase) and mechanical approaches (such as vigorous shaking, pipetting or scraping). The former hydrolyze the extracellular matrix (ECM) proteins to release individual cells, which considerably diminish the benefits associated with ECM proteins; while the latter, to a great extent, rupture cells leading to malfunction and cell death.[5,15] Non-invasive methods to harvest highly viable and undamaged stem cells have been subject to great demand to keep efficiency and functionality of stem cells.[16]

Several studies have investigated controlled growth and release of cells from TRP surfaces in biomedical applications. [5,15,17–19] Controlled attachment/growth and release of cells from TRP

surfaces is mostly explained in the term of hydrophobic/hydrophilic transition by temperature decrease.[20,21] However, it is more precise to relate the change in affinity of TRP coated surfaces towards cell/protein adhesion to change in hydration behavior of these surfaces.[22]

At a temperature above LCST (~32°C for both pNIPAAm and PVME), hydrogen bonds between water molecules and the amide (in pNIPAAm) or ether (in PVME) groups in the polymer chains become weak, susceptible to breakage and tend to expel from intramolecular structure of the polymer into the bulk water. This results in the collapse of the polymer chains in water, and the collapsed hydrophobic state promotes protein adsorption, cell attachment and subsequent proliferation. Decreasing temperature below LCST initiates hydration/swelling of the polymer chains due to the formation of hydrogen bonds with surrounding water molecules. In the swollen state, entropic repulsion of protein adsorption subsequently leads to release of intact cells accompanied by underlying ECM proteins.[8,23,24]

Several studies have used pNIPAAm as a common TRP to coat substrates to study controlled attachment and release of the cells.[25–28] Although PVME is a novel material with no adverse biological reactions and good mechanical strength, unlike pNIPAAm, very few studies have reported on biomedical applications of this polymer.[29] PVME is inexpensive and widely available. It is a non-ionic polymer that contains both hydrophobic (CH_3 and CH_2) and hydrophilic (-O-CH_3) groups. Below the LCST, hydrophilic ether oxygens form hydrogen bonds with water molecules leading to a single homogeneous hydrated phase, making PVME unfavorable for protein adsorption.[30] In addition, the non-ionic nature of PVME eliminates electrostatic forces between the protein/cell and the surface. Above the LCST, the hydrophobic interaction between apolar moieties becomes dominant, leading to the collapse of the PVME chains and a slightly hydrophobic PVME.[31,32] In addition, PVME is easily processable (due to its low glass transition

temperature, T_g ~ –28°C)[33] with fast shrinking behavior and no hysteresis for heating/cooling cycles.[32]

In this study, the potential of PVME serving as thermoresponsive substrates has been investigated, including a brief assessment on using it as a thermoresponsive culture support (TRCS) for rapid harvesting of hMSCs. In the two reported studies of employing PVME as TRCS, it was either blended with pNIPAAm to enhance cell detachment, or co-polymerized with maleic acid[34] and cell adhesion proteins (e.g., laminin)[29] to promote cell adhesion. The specific role of PVME in cell attachment/detachment was not clearly evaluated. In addition, the non-easily accessible electron beam technique was applied for their immobilization of PVME. In the present study, we investigated the processing conditions in utilizing 3-aminopropyltriethoxysilane (APTES), which forms a cross-linked network on a surface upon thermal treatment and has been shown to successfully retain a stable layer of pNIPAAm,[35–37] for immobilizing PVME on a hydroxylated surface such as glass. We examined the annealing temperature, annealing time and the PVME:APTES ratio. The retention of PVME on glass or silicon wafer substrates was verified by film thickness and water contact angles after thoroughly rinsing the sample with room temperature deionized (DI) water and also soaking the samples for 3 days in DI water at room temperature. All the retained PVME films showed thermoresponsive behaviors (TRBs), by water contact angles above and below the (LCST) of PVME (i.e., ~ 30°C). The PVME films fabricated via this simple entrapment method showed comparable switchable protein/cell adhesion behaviors as those of previous studies.[36,37] Additionally, PVME films (~70 nm after rinsing) showed rapid cell detachment similar to that of pNIPAAm films reported previously.[35,36,38] This inexpensive straight forward approach can be easily adopted by any laboratories, the mild processing conditions (e.g., ethanol or water as the solvent; processing temperature ≤ 80°C) also allow using

common polymers containing hydroxyl groups (e.g., cellulose, polyester, or oxidized polystyrene) as the substrates to expand its applications.

2. EXPERIMENTAL SECTION

2.1. Materials and equipment. Poly(methyl vinyl ether) (PVME, 50% solution in H_2O, 1.03 g/ml) and 99% (3-Aminopropyl) triethoxysilane (APTES) (Mw= 221 g/mol or 137 g/mol when fully hydrolyzed) were purchased from Sigma-Aldrich. 2-[Methoxypoly(ethyleneoxy)propyl] trimethoxy-silane (PEG-silane) was purchased from Gelest. Phosphate buffered saline (PBS) 1x was from Sigma–Aldrich. 30% hydrogen peroxide was from BDH and 98% concentrated sulfuric acid (H_2SO_4) was from VWR, and 200 proof ethanol, toluene, hexane, and hydrochloric acid (HCl) were from EMD. The probe liquids, methylene iodide (MI) and ethylene glycol (EG) were from Sigma, and DI water was purified in-house (with a conductivity of ~0.5 S/cm). Cells were cultured in Stemline mesenchymal stem cell expansion medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 4% (v/v) GlutaMAX from ThermoFisher, 1% of antibiotic antimycotic solution (100x) and 9 ng/ml recombinant human FGF-basic from PeproTech in a humidified 5% CO_2 atmosphere. Unless otherwise mentioned, all cell culture materials and also 1x trypsin/EDTA were purchased from Sigma-Aldrich. Normal human bone marrow derived mesenchymal stem cells (hMSC)s were purchased from Sigma-Aldrich. Albumin-fluorescein isothiocyanate (FITC-albumin) was purchased from Sigma-Aldrich. 1μm fluorescent negatively charged (carboxylate-modified) polystyrene particles were purchased from Life Technologies. Additionally, microscope glass slides were from VWR and silicon wafers (Si) were P type P<100> from Silicon specialist. Equipment were implemented in the present study include a spin coater (p-6000 Spin Coater, Specialty Coating System Inc., Indianapolis, IN), a

UV/Ozone cleaner (model 42, Jelight Company, Inc, Irvine, CA), analytical balances with an accuracy of 0.1 mg, a vacuum oven (VWR, Radnor, PA), its pump (Welch, Concord, MA) and water bath (ANOVA). A contact angle goniometer (Ramè-Hart Instrument Co., Netcong, NJ) with a CCD camera attached, an ellipsometer (Rudolph Instruments, Inc., Fairfield, NJ equipped with λ= 632.8 nm laser), a digital camera fitted with appropriate filters, an optical microscope (OM) with an eye-piece digital camera, an atomic force microscope (AFM) (Bruker Multimode AFM with Nanoscope V controller, Billerica, MA), and a Microplate Reader (Tecan Infinite 200, GMI - Trusted Laboratory Solutions, Ramsey, MN).

2.2. Surface immobilization of PVME. 1.25 cm × 1.25 cm glass slides and Si-wafers were cleaned using a freshly prepared piranha solution followed by copious DI water rinsing. The slide or wafer was dried with a stream of dry air, and then oxidized for 8 min in the UV/Ozone chamber. 2 wt% of APTES and PVME in 200 proof ethanol were prepared separately and then they were mixed to make PVME:APTES blends with mass ratios of 90:10 and 50:50. Each solution mixture was spin-coated (~100 µl solution flooding the sample surface) within 1 hour after preparation, on a freshly cleaned and oxidized Si-wafer or glass slide at a spin-speed of ~2000 rpm for 60 seconds. The spin-coated Si-wafers and glass slides were placed inside glass petri-dishes and then inside the vacuum oven to be annealed at 40, 60, 80, or 120 °C for one, two or three days. The annealed samples were removed from the oven, cooled and individually placed into the wells of 12-well plates and vacuum sealed prior to use. The non-annealed samples were also placed inside the wells of 24-well plates and vacuum sealed.

2.3. PEG-Silane surface preparation. The PEG-silane modified surfaces were prepared using the cleaned and oxidized glass slides. The glass slides were immersed in in a solution of PEG-silane in HPLC grade toluene (3 mM with 0.8 mL of HCl concentration/L) for 18 h at room

temperature. Consequently, the modified surfaces were sonicated twice in toluene and then twice in ethanol for 5 min to remove unreacted molecules. The modified substrates were then dried under air flow and stored under ambient conditions.

2.4. Characterization of retained PVME films. The films on Si-wafer were mainly used for thickness measurements and atomic force microscopy scans. The films prepared on glass slides were for measuring the water contact angles. The film thickness was measured via an ellipsometer with $\lambda = 632.8$ nm laser. The values were used to estimate the retention of the films on the surface. For PVME films and all PVME:APTES blend films, a refractive index of 1.46 was used to estimate the film thickness in air. For the thickness of APTES and silicon oxide in air, the refractive indices of 1.423 and 1.462 were used, respectively. The thickness of PVME:APTES blend films were measured at room temperature in three stages: after annealing, after thoroughly rinsing in room temperature DI water and after soaking in DI water for three days. For the thoroughly rinsed and 3 days soaked PVME:APTES blend films, advancing and static water contact angles were measured at 40°C and 25°C by placing the sample on a stage that allowed heating and cooling. After the stage reached to the set temperature (40°C or 25°C), the sample was placed on the stage and allowed to equilibrate for ~30 seconds. Then, a water drop (~10 μl) was placed on the sample, with the needle in the drop, and more water was slowly added until the drop was ready to advance, at which point the image was captured. The time from adding the drop to taking the image was ~1 min. After two advancing measurements, the needle was withdrawn, and the drop (~20 μl) was allowed to sit on the sample for ~30 seconds before the image was taken for the static contact angle. To obtain the LCST temperature of the retained PVME:APTES blend films, a thoroughly rinsed film was placed on the stage, and the stage was cooled down from 42°C to 23°C or heated up 23°C to 42°C at a rate of ~1 °C/min. The image of the advancing water contact angle at the end

of each minute was captured. The contact angles were measured from the captured images using ImageJ software.

2.5. Particle attachments. The particle attachment experiment was carried out on 2% PVME:APTES blends 90:10 and 50:50, PEG-silane and 1% APTES. A 100 µl of negatively charged polystyrene particle solution (7×10^6 particles/mL in 0.1x PBS) was deposited on each sample surface and the adsorption was carried out for 1 hour at room temperature (25°C) and at 37°C. Subsequently, the samples were washed 2 times with 1x PBS and then DI water. In order to evaluate the particle attachment, randomly selected 10 regions were imaged under the fluorescent light on two samples and the particles were counted using ImageJ software and then the values were normalized by the image area.

2.6. Protein adsorption. The 2% PVME:APTES blends 90:10, 50:50 and 20:80, PEG-silane and 1% APTES substrates were rinsed with DI water and dried with a stream of air flow and then covered with a 100 µl of FITC-albumin solution (1 mg/mL in 1x PBS). The effect of temperature on protein adsorption behavior of PVME films was studied in 3 ways: 1) samples were incubated in albumin solution at room temperatures for 2 h; 2) samples from the first set underwent a temperature increase to 37°C and the samples were soaked for another 2 h; 3) The samples from the second set were rinsed with DI water and then incubated in 1x PBS at room temperature for 2 h. All samples were rinsed thoroughly twice with the test temperature 1x PBS and then DI water. The samples were dried with airflow and then imaged using a fluorescent microscope. To evaluate the FITC-albumin adsorption, the mean-gray-values of adsorption images were quantified by ImageJ. The mean-gray-values were obtained from 10 images of randomly selected regions on two samples.

2.7. Cell adhesion and detachment on PVME films. Cell adhesion experiments were carried out by culturing hMSC on 2% PVME:APTES (50:50) thin films. First the film coated glass slides were rinsed with DI water in room temperature and dried with stream of air flow. Then the samples were sterilized under UV light for 15 minutes and placed in 35 mm petri dishes. The cells were harvested from tissue culture flasks with 1x trypsin/EDTA. Subsequently, hMSC were seeded at a density of 10k cells/cm^2 on the film coated glass slides and incubated in hMSC expansion medium at 37°C and a humidified 5% CO_2 atmosphere for 1 day or 3 days. The cell detachment process was observed via a microscope-video system and using a 10x phase objective in room temperature. A sequence of images was captured in a fixed position with 30 seconds time intervals for 1 hour. Each sample had one replicate.

2.8. Statistical analyses. Experimental results were presented as mean ± standard deviation. A significance level of $\alpha = 0.05$ was considered for all statistical analyses performed in Minitab 17 software. The differences between the groups for each temperature (25°C and 37°C) were evaluated using one-way analysis of variance (ANOVA) and multiple group comparisons using a post hoc Tuckey's range test. The pair of data was considered statistically different when p-value < 0.05.

3. RESULTS AND DISCUSSION

3.1. PVME film retention. Similar to the previous study of retaining pNIPAAm films by the APTES network,[36] spin-coated PVME films without APTES, or with APTES but without thermal annealing (i.e., annealed at room temperature) were easily removed when rinsing the samples with room temperature DI water (Figure S1). For all the PVME:APTES films (90:10 and 50:50) annealed for one, two, or three days at temperatures from 40°C to 120°C, film retention (see Figure

1 for those annealed at 120°C for 1 day) was observed after rinsing with and soaking the samples for three days in room temperature DI water.

Regardless the processing condition, the thickness of post thermal annealed films, before rinsing, only depended on the initial PVME:APTES solution concentration, which was ~126 nm and ~76 nm, respectively, for the PVME:APTES 90:10 and 50:50 blend films. The retained PVME films, i.e., after room temperature water rinsing and soaking to remove the water soluble PVME, however, varied with PVME content in the blend, annealing temperature and annealing time. The 90:10 blend films were generally thicker than the 50:50 blend films: ~67% more after annealing, ~37% more after rinsing, and ~55% more after soaking.

As illustrated in Figure 1 and Figure S1, thermal annealing was necessary for the retention of the PVME films on the surface. Thermal annealing allowed the APTES molecules to cross-link and form a tight network to entrap the PVME chains, thus retaining a PVME film on the surface.[36] At room temperature, hydrolyzed APTES molecules formed oligomers and deposited to the surface to form a relatively loose network, which was unable to firmly entrap/interlock polymer chains,[39] resulting in no film retention. When the annealing temperature increased to 40°C, some PVME retention (13–24% of the post-annealed film after soaking) was noticed (Figure 2). The retention increased with the increase of PVME in the blend or the annealing time from one day to two days, but no further increase with a longer annealing time (e.g., three days). Further improvement in retention was observed with the increase of annealing temperature, especially for films after soaking in room temperature water for 3 days. For 3 days of annealing at a temperature of 60 °C, 34% and 31% of 50:50 and 90:10 blend films, respectively, were retained; the retention increased to over 80% for the 50:50 blend and over 65-75% for the 90:10 blend when annealed at 80 °C and 120 °C. For an annealing temperature of 80 °C or 120 °C, retention of PVME films on

the surface by the APTES network after annealing for two days or three days was basically the same, whereas with one day of annealing, a slightly less retention (at 80 °C: 55–59% retained after soaking, at 120 °C: 66–75% retained after soaking) was observed. These results also demonstrated that a shorter annealing time was sufficient when the samples were annealed at a higher temperature. At 120 °C, annealing the samples for one day was adequate to retain > 85% of the film that could be retained with a longer annealing time.

Less film retention at 40 °C and 60 °C as compared to higher annealing temperatures was likely the result of not being able to form a tight APTES network at these temperatures. Annealing APTES at lower temperatures (< 80 °C) has been reported to leave some hydroxyl groups in the hydrolyzed APTES molecules unreacted, causing APTES network not to be fully cross-linked. [40,41] Nevertheless, some crosslinking for APTES at an annealing temperature of 65 °C has been evidenced. [40,41] In addition, at 40 °C, hydrogen bonds between ether groups in PVME and amine and hydroxyl groups in hydrolyzed APTES might facilitate PVME retention by APTES. Furthermore, the low T_g of PVME (-28 °C)[42], hence high mobility of the PVME chains, could potentially allow an ease penetration of PVME, at 40 °C and 60 °C, into the APTES network to retain PVME chains on the surface. Annealing at elevated temperatures (> 80 °C) increased the cross-linking between APTES molecules and tightened the APTES network that led to less swelling after soaking in water, making it more difficult for the PVME chains to be pulled out during soaking, [36,43] hence a greater PVME retention for samples annealed at these evaluated temperatures.

Annealing at 120 °C for 24 hours led to a sufficient APTES cross-linking,[43] thus a longer than 24 h of annealing did not significantly improve film retention. The cross-linking of APTES at the polymer/substrate interface was the result of segregation of enough APTES molecules to the

interface through PVME matrix due to the small size of APTES molecules and the higher surface energy of APTES (~40 mJ/m^2) than that of PVME (~30 mJ/m^2).[33] The migration of APTES molecules through the polymer melt to segregate at the polymer/substrate interface had been confirmed in our earlier study using pNIPAAm and APTES blend.[36] One advantage of using PVME over pNIPAAm is its low T_g (-28 °C vs. 135 °C for pNIPAAm), which allows the films to be processed at temperatures as low as 40 °C to achieve the entrapment and retention of PVME in an APTES network. Since the migration of APTES molecules through the low viscosity PVME melt would occur easily, as long as the temperature is sufficient to cross-link the segregated APTES molecules at the PVME/substrate to form a network for entrapping PVME chains, PVME can be retained on the surface. Once a tight APTES network is formed, e.g., probably within the first day of annealing at 80 and 120 °C, increasing the annealing time did not necessarily reinforce the APTES network to result in more film retention. A better retention of the PVME was also observed using a higher APTES ratio in the blend when annealed at 80 or 120 °C (Figure S2).

3.2. Thermoresponsive behaviors of the retained PVME films. To assess the thermoresponsive behaviors of the retained PVME films, water contact angles were first measured on the films, and then particle attachment, protein adsorption and cell attachment/detachment experiments were carried out. The results are summarized in this section.

3.2.1. Water contact angles. The lower critical solution temperature (LCST) of retained PVME:APTES blend films (90:10 and 50:50) was first measured. The LCST of the PVME:APTES 90:10 and 50:50 was found to be 32-34°C (see Figure S4), which was close to the value (30–32°C) of the PVME in an aqueous solution (see S1 Supporting Information). Figure 3 shows that the advancing water contact angles of the retained PVME films resulted from different processing conditions of the PVME:APTES blends (90:10 and 50:50).[35,36] The advancing water contact angles

(θ_A) at a temperature of ~40 °C (> LCST of PVME) on the retained 90:10 and 50:50 blend films were found to be significantly higher (~14°, p= 0.00 for 90:10 blend films, and ~21°, p=0.00 for 50:50 blend films) than those measured at a temperature of 23 °C (< LCST of PVME), verifying the hydrophilic to hydrophobic transition and thermoresponsive behavior (TRB) of the retained PVME films. Water contact angle on PVME films at 20 °C was estimated to be 74.8° [44] using the reported surface energy of PVME (31.8 mJ/m^2)[3] (see S1 Supporting Information for more details), which was comparable to the value measured on PVME:APTES 50:50 films (~72°) (Figure 3b). The slightly more hydrophobic 50:50 blend films than 90:10 blend films (θ_A of ~72° vs. ~66° at room temperature)[45] could be due to slightly more APTES molecules, containing hydrophobic alkyl chains, remained on the top surface of the retained films. At a temperature below LCST, PVME films were in a swollen state and the extended PVME chains could cover the APTES layer, resulting in a more hydrophilic behavior.

3.2.2. Particle attachment. Figure 4 and Figure 5 illustrate the attachment results of carboxylate (-COOH) functionalized polystyrene latex particles on various surfaces. At room temperature (< LCST of PVME), PVME films resulted from 90:10 and 50:50 PVME:APTES blends showed low particle attachments (~1 particle/mm^2 and ~9 particle/mm^2, respectively), similar to that of the low fouling PEG-silane surface (~2 particle/mm^2), while the APTES surface had the highest attachment (~373 particle/mm^2). At 37 °C (> LCST of PVME), the attachments on PVME films from 90:10 and 50:50 PVME:APTES blends were ~536 particle/mm^2 and ~808 particle/mm^2, respectively, orders of magnitude greater than particle attachment at room temperature. Whereas, there were only slight increases on both PEG-silane (~10 particle/mm^2) and APTES films (563 particle/mm^2) from those at room temperature. The films from PVME:APTES

50:50 showed higher particle attachment than that on films from PVME:APTES 90:10 (~1.5×). This could be related to the higher content of APTES in the 50:50 PVME:APTES blend and a slightly more APTES molecules remained on the top surface of the retained films after the annealing process. $–NH_2$ groups of these remained APTES molecules could potentially be exposed to interact with the –COOH group in the PS particle. Both electrostatic and van-der Waals interactions would enhance with the exposure of these $–NH_2$ groups, leading to a greater attachment. An additional reason could be surface roughness, a higher roughness was expected to result in more attachment. Based on the atomic force microscopy (AFM) scans (more details can be found in Supporting Information, Figure S3), the root mean square roughness (R_q) of the 50:50 PVME:APTES blend film (R_q = 1.92 nm for a 2 μm × 2 μm scan) was found to be higher than that of 90:10 PVME:APTES blend film (R_q = 0.84 nm for a 2 μm × 2 μm scan).

3.3.3. Protein adsorption. At room temperature, due to the hydrophilic state of PVME surfaces (90:10 and 50:50), a lower fluorescent intensity of adsorbed FITC- albumin (~25% and ~31%, respectively) was observed, while it was still higher than that on the PEG-silane surface (~0%). Additionally, at room temperature, albumin adsorption increased with the amount of APTES in the PVME:APTES blend. As mentioned in the particle attachment section, PVME from 50:50 PVME:APTES blend would have more $–NH_2$ groups from APTES molecules that could potentially expose to enhance interactions with proteins. To verify this speculation, retaining PVME using a PVME:APTES 20:80 mixture was prepared, and indeed a higher protein adsorption (~55%) as compared to the other two PVME surfaces (from 90:10 and 50:50 blends) was detected. Also, as expected, the APTES surface showed the highest protein adsorption (~100%). This also illustrated the greater contribution of amine group from APTES than surface roughness in protein adsorption, since the roughness of 50:50 PVME:APTES blend film (R_q = 1.92 nm) was greater

than both 20:80 PVME:APTES blend (R_q = 1.29 nm) and APTES (R_q = 1.25 nm), but the protein adsorption on the 50:50 PVME:APTES blend was lower.

By increasing the temperature to 37 °C, PVME retained using 90:10, 50:50 and 20:80 PVME:APTES blends achieved a statistically significant increase ($p < 0.05$) in protein adsorption (Figure 6). The fluorescent intensities on PVME films from 90:10, 50:50 and 20:80 blends were ~69%, ~96% and 70%, respectively. The increase in hydrophobicity of the PVME surfaces (Figure 3), with the collapse of the PVME chains, promotes affinity of the proteins to the PVME surfaces [46,47], which led to a higher protein adsorption at 37 °C. Also at 37 °C, a higher APTES content in the retained PVME film from the 50:50 blend (Figure 3) and the exposure of remained APTES molecules on the top film surface to the surrounding as the PVME chains collapsed could be the reason why this surface showed a dramatic increase in protein adsorption compared to room temperature. On the contrary, PEG-silane and APTES surfaces showed no significant change in protein adsorption by increasing the temperature to 37 °C.

In order to examine the reversibility of protein adsorption on the retained PVME films, albumin adsorption with temperature cycles were performed on these films (Figure 6). The results showed that by decreasing the temperature below LCST after the adsorption took place at 37 °C, the proteins desorbed partially from the PVME films retained from 90:10 (~30% desorption) and 50:50 (~38% desorption) blends. PVME films from the 50:50 blend showed slightly more desorption (by ~27%) as compared to the 90:10 blend. By increasing temperature above LCST, as the PVME chains collapsed, the -NH_2 groups could again be exposed to the protein solution to result in more protein adsorption for the films from the 50:50 PVME:APTES blend. Consequently, during the desorption cycle, as the temperature decreased, the PVME chains extended and covered the exposed –NH_2 groups in APTES to result in a higher protein desorption from the PVME

retained film of the 50:50 blend. The PVME film retained from the 20:80 blend, however, did not show a noticeable protein desorption by decreasing temperature, indicating the presence of more non thermally reversible APTES molecules hinders the thermo-reversibility of small amount of PVME retained. The partial reversibility of protein adsorption on PVME surfaces could be due to incomplete recovery from the hydrophobic state to the hydrophilic (swollen) state by decreasing temperature below LCST. The change in protein conformation and/or orientation might also decrease the protein's activity, leading to partial reversibility.[8]

3.2.4. Switchable cell Adhesion on PVME surfaces. hMSCs were cultured on PVME films retained using the PVME:APTES 50:50 blend to observe individual cell attachment/detachment behaviors. Since PVME showed almost no toxicity towards hMSCs as a retained film (details in the S1 Supporting Information file, Figure S5), after one day of cell culture, hMSC cells grew and spread, exhibiting their normal flat and spindle-like morphology on the PVME films, similar to those on the polystyrene cell culture dishes (Figure 7). Figure 8 presents images of cell detachment at different time points. For 1-day cell culture, the cell detachment started, by changing cell morphology, in 5 minutes after the media reached room temperature. After 12 minutes, the cells showed a less spread morphology and some of them were already rounded up. After 17 minutes, most of the cells lost their anchorage points and started moving. Finally, after 25 minutes, all the cells turned into a round morphology. Of note, almost all the cells detached after gently shaking the dish for 10 seconds (see the video clip in the supplemental data section). No detachment of cells from the polystyrene cell culture dish at room temperature was observed even after 1 hour. For samples with three-day cell culture, the cells mostly detached and started moving at minute 14, and within 30 seconds, all cells detached as a sheet from the surface. The detachment occurred faster for three-day cultured cells than for one-day cultured cells. A

longer proliferation period (3 days) resulted in more cells in contact with each other, leading to a simultaneous contraction that causes accelerated cell detachment.[48] Similar hMSCs attachment/detachment behaviors were observed for PVME surfaces compared to the pNIPAAm retained on a surface by the APTES network (more details can be found in the Supporting Information file, Figure S6).

4. CONCLUSION

In this study, we investigated the conditions in forming a cross-linked APTES network on a surface upon thermal treatment to retain a stable layer of PVME. The blend of PVME:APTES in Ethanol was spin-coated and then thermally annealed from 120°C down to 40°C for one to three days. The PVME film retention was evaluated by rinsing with and then soaking in room temperature DI water. A higher annealing temperature led to more film retention in both 90:10 and 50:50 PVME:APTES blends, irrespective of annealing time. The polymerized and cross-linked APTES molecules formed a network when underwent thermal annealing. Advantageous over pNIPAAm, PVME, having a much lower T_g, allows an easier migration of APTES molecules through PVME matrix to segregate at polymer/substrate interface, provides sufficient chain mobility to penetrate into APTES network that was chemically grafted on the surface and to enhance interactions between PVME chains and APTES molecules. As a result, PVME film retention by APTES at temperatures as low as 40 °C was observed. Regardless of processing conditions, all retained PVME films exhibited thermoresponsive behavior, assessed and confirmed by water contact angle, particle attachment, protein adsorption, and cell attachment/detachment. The results from particle attachment and protein adsorption provide insights into the design of better thermoresponsive antifouling surfaces for applications such as membrane separation and protein sorting.

Furthermore, cell attachment/detachment of hMSCs on the retained PVME films by a simple switching of temperature suggests the potential of PVME films for non-invasive harvesting of stem cells in the fields of tissue engineering and regenerative medicine.

ASSOCIATED CONTENT

S1 Supporting Information

The lower critical solution temperature (LCST) determination of PVME solution and retained films; digital images showing no film retention of PVME:APTES (90:10 and 50:50) films (spin-coated from 2 wt.% solution) without thermal annealing and also PVME films without APTES but with annealing; FTIR-spectra of the PVME:APTES 90:10 and 50:50 blends showing the presence of APTES in PVME, but no specific chemical interactions; atomic force microscopy (AFM) topography scans of APTES and various PVME:APTES blend films; estimation of water contact angle on PVME films at room temperature; cytotoxicity of PVME towards hMSCs; cell attachment/detachment on APTES retained pNIPAAm films;

S2 Supporting Information

A movie clip showing individual human mesenchymal stem cells (hMSC) detachment at room temperature from retained 2% PVME:APTES 50:50 film after 1 day of cell culture. Images in Figure 8a were taken from this clip.

S3 Supporting Information

A movie clip showing human mesenchymal stem cells (hMSC) detachment at room temperature from retained 2% PVME:APTES 50:50 films after 3 days of cell culture. Images in Figures 8b were taken from this clip.

ACKNOWLEDGMENTS

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Graphic for manuscript

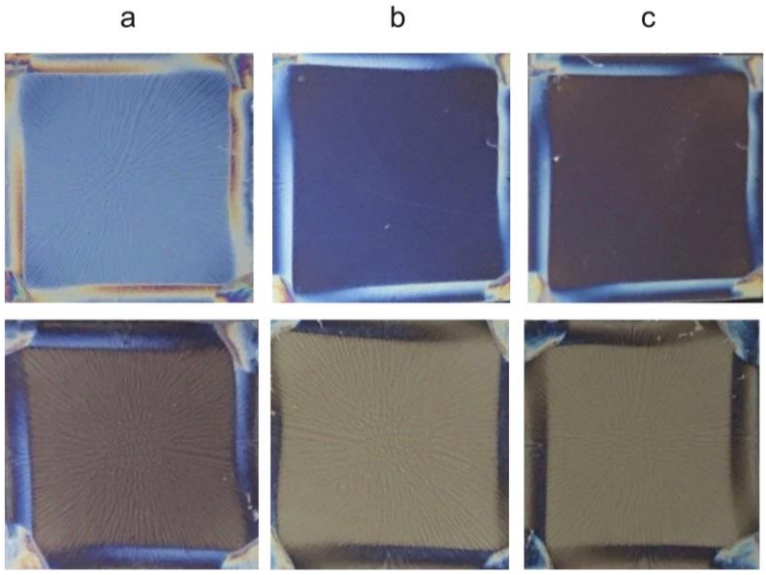


Figure 1. Digital images of 90:10 PVME:APTES films (top) and 50:50 PVME:APTES films (bottom) spin-coated on silicon wafer (1.25 cm × 1.25 cm) from 2 wt% solution in ethanol. (a) Post-annealed (top: light blue, thickness ~126 nm; bottom: dark brown, thickness ~77 nm). (b) Rinsed by room temperature DI water (top: dark blue, thickness ~111 nm; bottom: light brown, thickness ~72 nm). (c) Soaked in room temperature DI water for 3 days (top: dark brown, thickness ~84 nm; bottom: light brown, thickness ~56 nm).

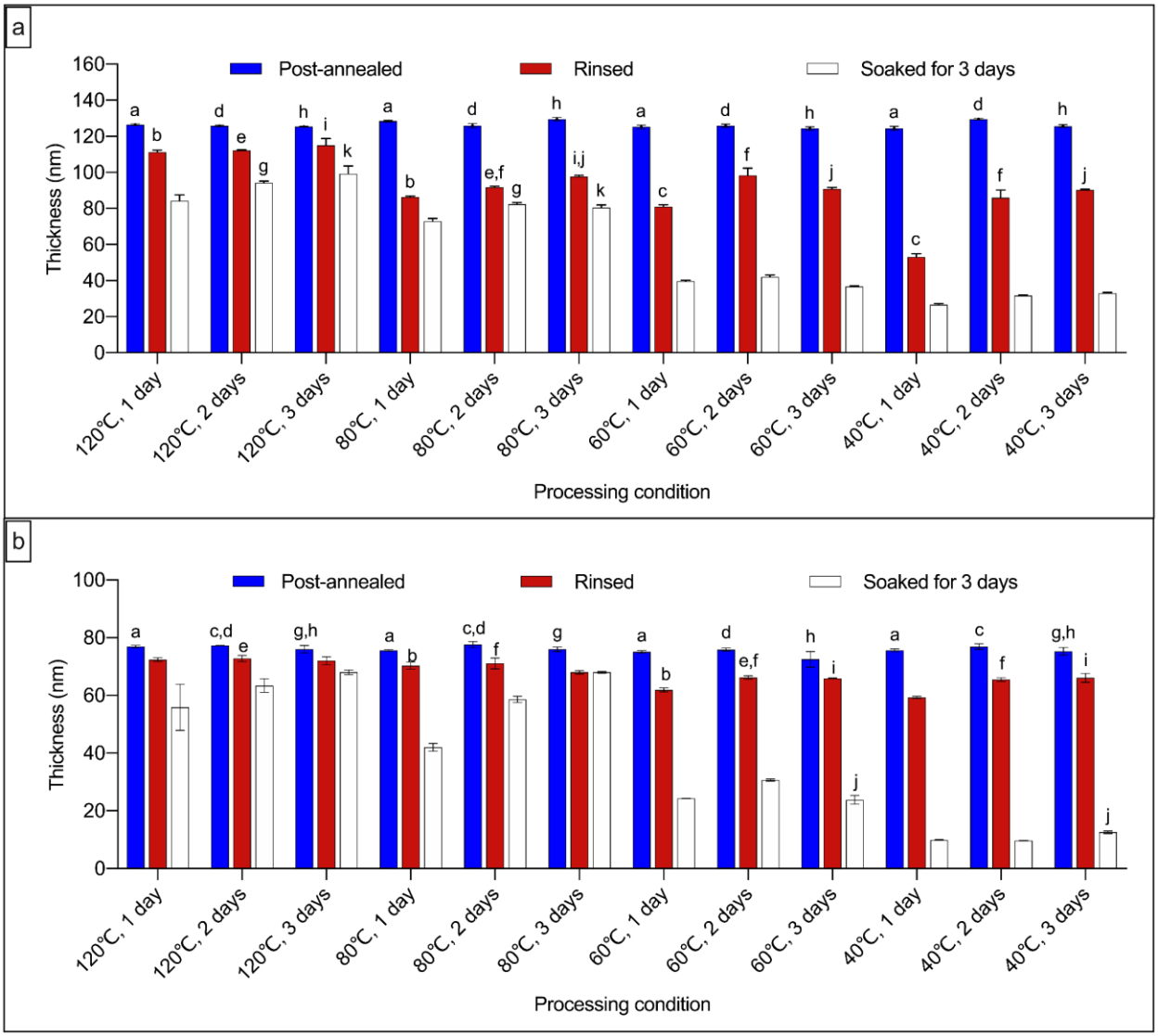


Figure 2. The film thickness at different processing conditions measured at three states: post-annealed, rinsed with room temperature DI water and soaked in room temperature DI water for 3 days. 2 wt.% PVME:APTES (a) 90:10, and (b) 50:50 blend films. The bars with the same annealing time and state that share the same letter are not significantly different ($P>0.05$).

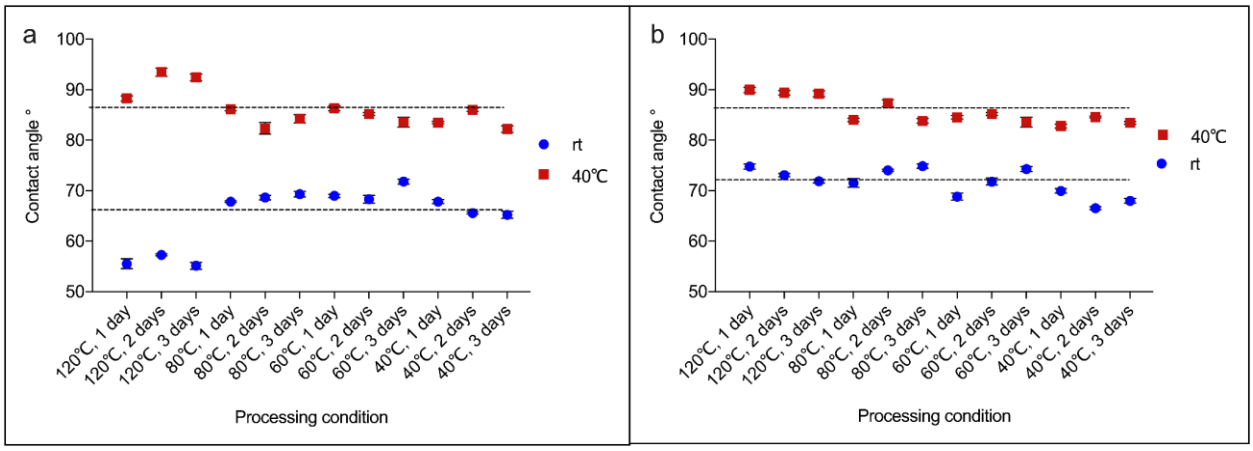


Figure 3. The advancing water contact angles (θ_A) above and below LCST (25 °C and 40 °C) of 2% PVME:APTES (a) 90:10, and (b) 50:50 blend films annealed for different time lengths (one, two or three days) in a vacuum oven and with different annealing temperatures (120- 40℃). The films were rinsed by DI water at room temperature to remove non-retained layer before measuring the water contact angle.

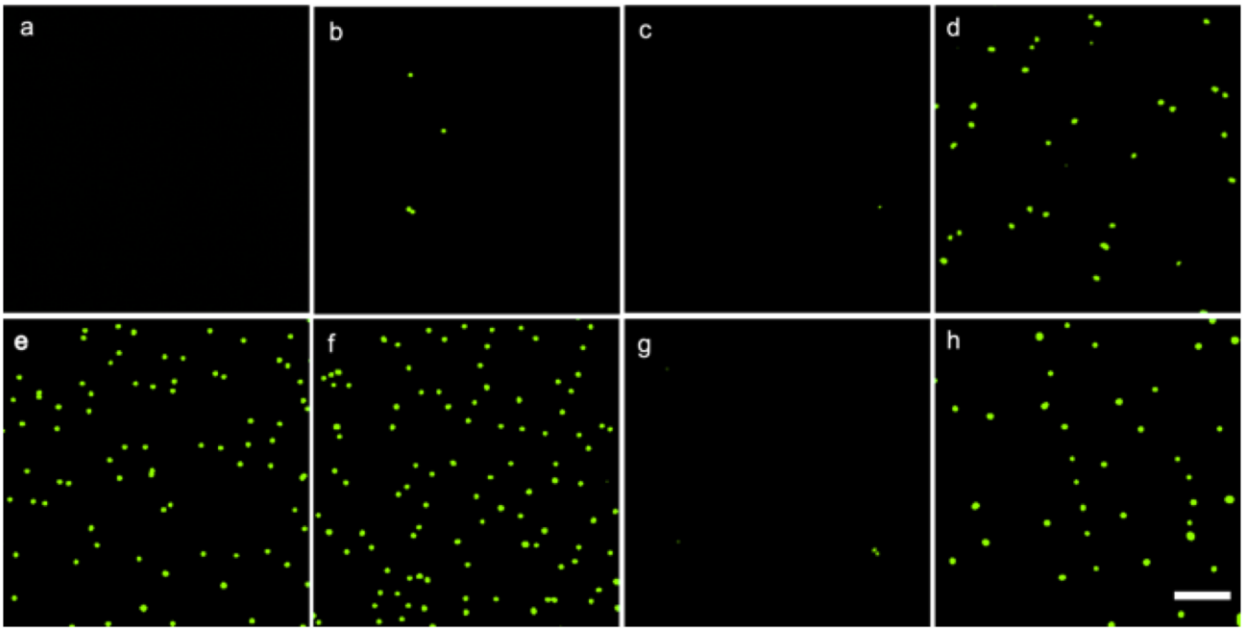


Figure 4. Fluorescent microscope images of negatively charged particle attached on (a) and (e) 2% PVME:APTES 90:10. (b) and (f) 2% PVME:APTES 50:50. (c) and (g) PEG-silane. (d) and (h) APTES at (top) 25 °C, and (bottom) 37 °C showing thermoresponsive behavior of PVME films. The scale bar in each image is 25 μm.

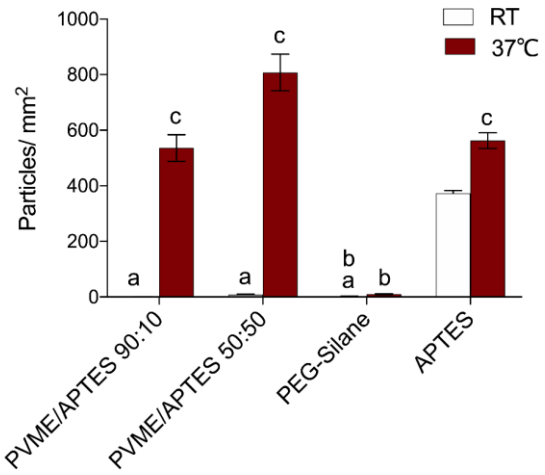


Figure 5. Bar graph showing quantitative data of the negatively charged particles attached on 2% PVME:APTES 90:10 and 50:50, PEG-silane and APTES at 25 °C and 37 °C. The bars that share the same letter are not significantly different ($P > 0.05$).

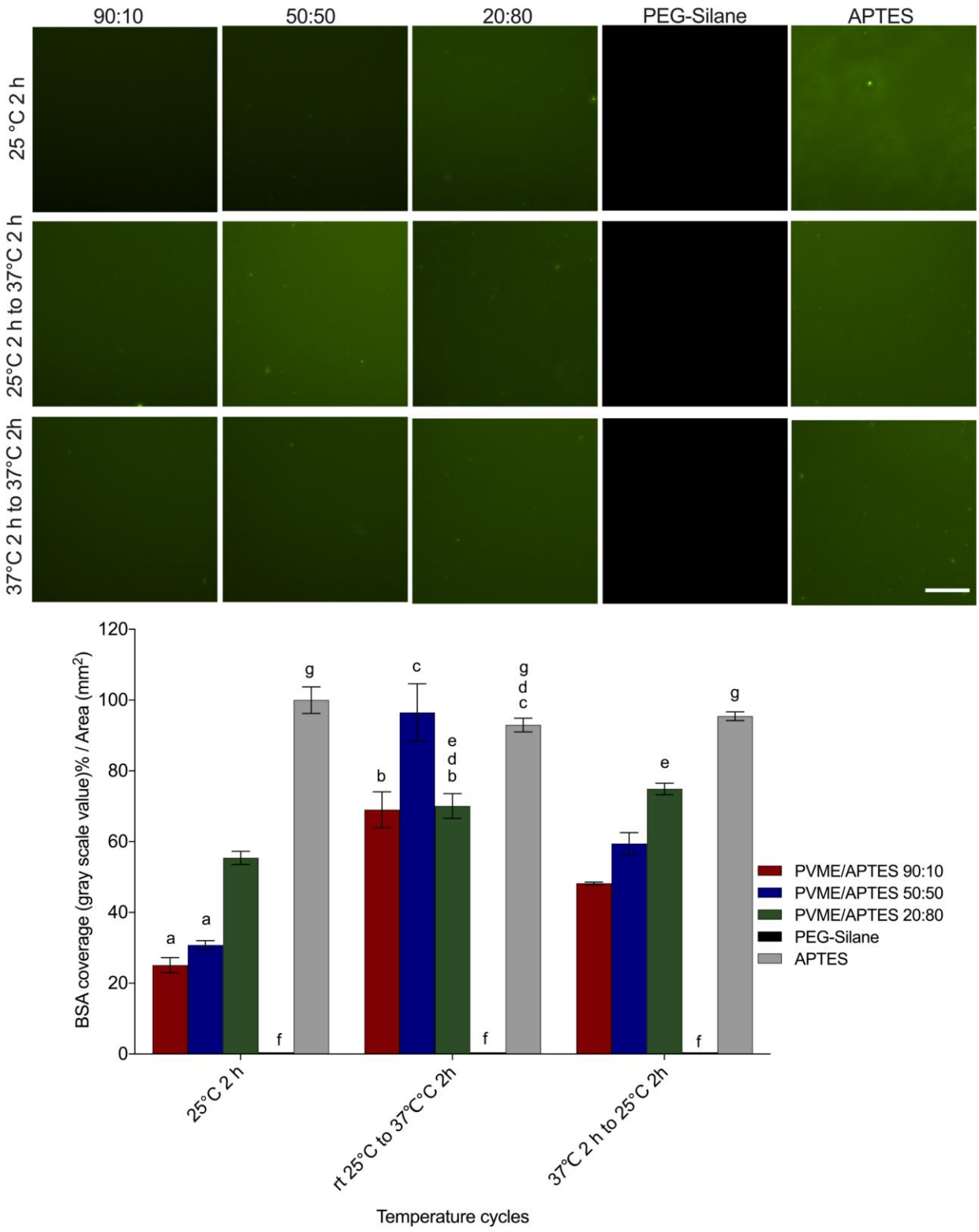


Figure 6. (Top) Fluorescent images of Bovine serum albumin (BSA) adsorption through

temperature cycles on 2% PVME:APTES 90:10 and 50:50, PEG-silane and APTES at 25 °C and 37 °C. Albumin adsorption is partially reversible on PVME films, but not on PEG-silane and APTES surfaces. (Bottom) Bar graph illustrating albumin adsorption. The bars that share the same letter are not significantly different ($P > 0.05$).

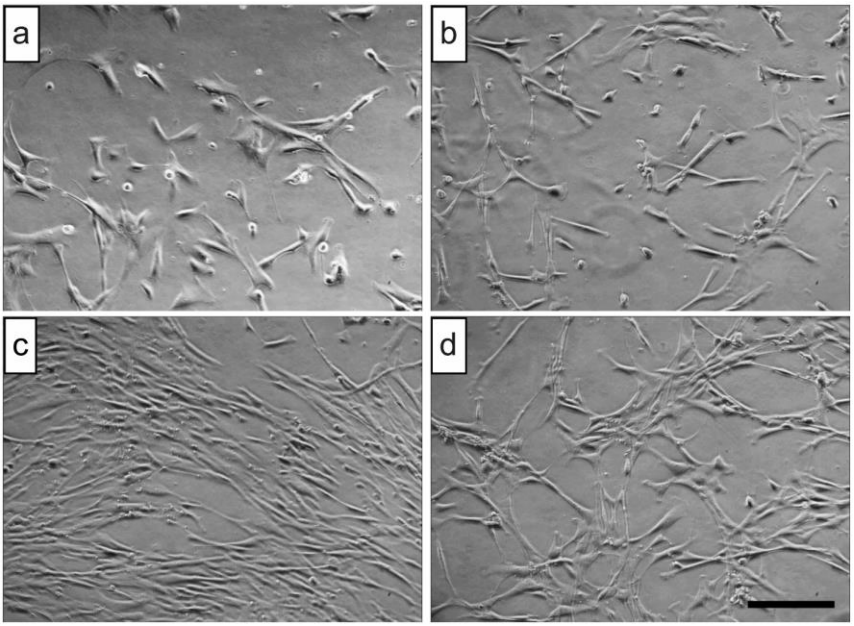


Figure 7. Human mesenchymal stem cells (hMSCs) seeded on (a) tissue culture polystyrene dish for 1 day. (b) 2% PVME:APTES 50:50 blend film on glass for 1 day. (c) tissue culture polystyrene dish for 3 days. d) 2% PVME:APTES 50:50 blend film on glass for 3 day with a density of 6000 cells/cm^2. The scale bar is 200 μm.

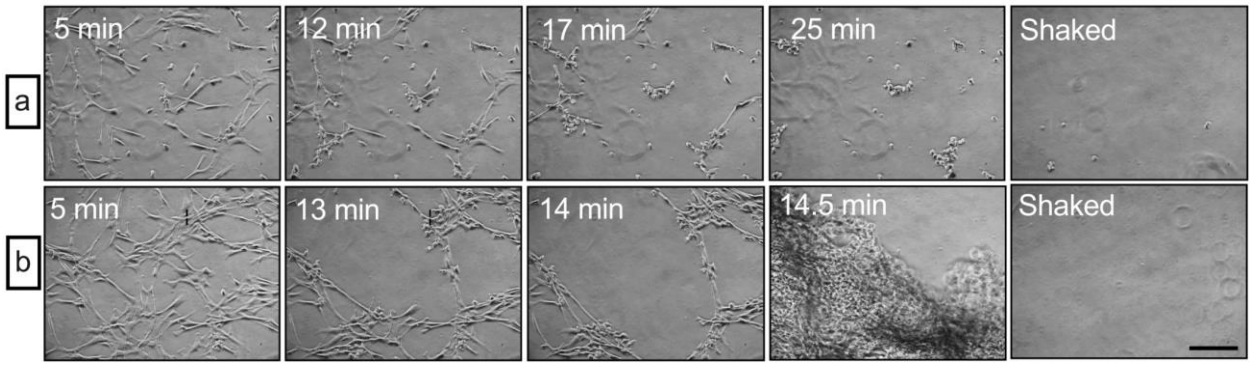
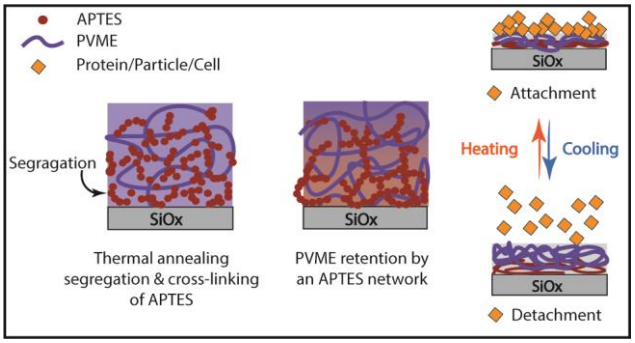


Figure 8. Phase contrast images of cell detachment captured on different time points from on 2% PVME:APTES 50:50 films (annealed at 80 °C for 1 day) after (a) one day, and (b) three days of culture. The dish was placed on microscope stage at room temperature. The temperature of the medium reached to room temperature after 5 minutes, at which point, the cell detachment started. Scale bar is 200 μm.



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