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Hydrophilic polyurethane matrix promotes chondrogenesis of mesenchymal stem cells★

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Abstract

Segmental polyurethanes exhibit biphasic morphology and can control cell fate by providing distinct matrix guided signals to increase the chondrogenic potential of mesenchymal stem cells (MSCs). Polyethylene glycol (PEG) based hydrophilic polyurethanes can deliver differential signals to MSCs through their matrix phases where hard segments are cell-interactive domains and PEG based soft segments are minimally interactive with cells. These coordinated communications can modulate cell–matrix interactions to control cell shape and size for chondrogenesis. Biphasic character and hydrophilicity of polyurethanes with gel like architecture provide a synthetic matrix conducive for chondrogenesis of MSCs, as evidenced by deposition of cartilage-associated extracellular matrix. Compared to monophasic hydrogels, presence of cell interactive domains in hydrophilic polyurethanes gels can balance cell–cell and cell–matrix interactions. These results demonstrate the correlation between lineage commitment and the changes in cell shape, cell–matrix interaction, and cell–cell adhesion during chondrogenic differentiation which is regulated by polyurethane phase morphology, and thus, represent hydrophilic polyurethanes as promising synthetic matrices for cartilage regeneration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.msec.2015.05.043>.

Keywords

Polyurethane; Gel; Mesenchymal stem cell; Chondrogenic differentiation

1. Introduction

Matrix guided cell-instructive cues are crucial for biomaterial based tissue regenerative applications. Synthetic materials should mimic the hierarchical architecture of native extracellular matrix to provide these cues to cells. In particular, biomaterial based strategies for cartilage regeneration are focused on developing provisional synthetic replacements which have structure and function resembling the native cartilage matrix and can support the cells in organizing into functional tissues. Native cartilage matrix is highly hydrated with 60–80% water content and the solid content is primarily biphasic with type II collagen and proteoglycans in a mesh-like network structure [1,2]. Chondrocytes derived from mesenchymal stem cells (MSCs) are resident cells of cartilage and are crucial for maintaining the extracellular matrix. These features contribute to the biophysical and mechanical properties of the cartilage matrix. Since cartilage exhibits limited intrinsic healing capacity, synthetic biomaterials are used to deliver therapeutically relevant cells including MSCs. Owing to the gel like architecture of native cartilage matrix, biomaterial based approaches for cartilage regeneration are focused on synthetic, semi-synthetic, and natural materials which can form gels with a high water content. Most widely used synthetic hydrogels are based on polyethylene glycol (PEG) while natural hydrogels are based on hyaluronic acid and alginate [3–7]. Gel-based materials are also preferable due to their ability to maintain the rounded morphology of cells, often considered a major factor for chondrogenesis of MSCs [8,9]. However, most of these gels do not mimic the biphasic structure of cartilage matrix. In addition these gels require chemical crosslinking to confine the cells within the matrix in a three-dimensional environment.

To address these features, we envisioned that segmental polyurethanes (PUs) with hydrophilic character can present a biphasic structure and form a gel-like architecture to produce a compatible synthetic matrix for chondrogenic differentiation of MSCs. PUs are essentially biphasic with soft and hard segments and segmental interactions. It is primarily H-bonding and electrostatic interactions drive the assembly of PU segments into a nanophase morphology [10–12]. Recently, our studies have shown that PU nanophases provide matrix-guided signals to control cell-fate by stimulating specific interactions [13]. However, these polyurethanes were unable to form gels with high water content due to their hydrophobic character. Therefore, in this study we aimed to use PEG based PUs which can form gel-like structures due to their high water absorption. Owing to the biphasic morphology of PUs and the ability to form hydrogels, PEG-PUs may mimic the functional characteristics of native cartilage matrix. Furthermore, PU gels do not require chemical crosslinking for stability as hard segments can assemble into distinct nanophasic structures due to non-covalent molecular interactions and, thus, allow hard segment domains to act as physical crosslinks between the soft segments. To assess, the ability of PEG based PUs to control MSCs toward chondrogenic lineage, we utilized PUs with PEG as the soft segment with aliphatic diisocyanates and L-tyrosine-based dipeptide chain extender as hard segment

[14]. By altering PU structure, we investigated the role of biphasic PU structure in controlling MSCs for chondrogenic differentiation of MSCs. These PUs are also degradable and, therefore, can act as a temporary synthetic analog for cartilage regeneration [15]. Thus far, use of PUs for cartilage regeneration has been focused on porous scaffolds which cannot mimic the highly hydrated gel-like structure of the native cartilage matrix [16]. In this context, this approach represents advancement toward utilizing PUs as gels for chondrogenesis of stem cells.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG) with number average molecular weight of 1000 was purchased from Sigma Aldrich and used after vacuum drying at 50 °C for 3 days to remove moisture. All other chemicals and solvents were purchased from Sigma Aldrich (MO) and were used as received unless otherwise noted. Desaminotyrosine tyrosyl hexyl ester (DTH) was synthesized according to a literature procedure [17]. Coverslips were purchased from Fisher Scientific (PA). Bone marrow derived MSCs were purchased from Texas A&M Health Science Center College of Medicine (supported through a grant from NCRR of the NIH). Cell culture medium (α -MEM), Alamar blue and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen, CA. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, GA, F-actin and focal adhesion staining kit (FAK100) from Millipore, MA, DNA quantification kit 'Quan-iT Picogreen' kit from Invitrogen, mouse anti-aggrecan from Santacruz Biotechnology, mouse anti-cadherin-11 from R&D systems and fluorescently labeled FITC and TRITC conjugated secondary antibodies from Millipore, MA were used. Collagen I and collagen II immunohistological staining was performed with commercial staining kits from Chondrex, Inc., WA and GAG and collagen II quantification was performed with quantification kits from Astrate Biologics, VA.

2.2. Polymer synthesis and substrate preparation

Polyurethanes were synthesized by a two-step process as described in prior publications [14]. Briefly, polyethylene glycol (PEG) and hexamethylene diisocyanate (HDI) or 4,4'-methylenebis(cyclohexyl isocyanate) (HMDI) (1:2 molar ratio) were reacted at 100 °C in dry DMF for 3 h in the presence of tin-2-ethyl hexanoate (0.1 mol%) as catalyst to form the prepolymer. After 3 h the reaction mixture was cooled to room temperature and DTH was added to it (PEG to DTH molar ratio was 1:1). The reaction mixture was stirred at 80 °C overnight. After cooling the reaction mixture to room temperature, it was poured into saturated sodium chloride solution and cooled in an ice bath to precipitate the polymer. Precipitated polyurethanes were filtered, washed with distilled water, and dried under vacuum at room temperature for 12 h. To form PU gels, precipitated polyurethane in aqueous media was aggregated by centrifugation to form a gel with entrapped water.

1 wt.% solutions of polyurethanes were prepared by dissolving the polymer in chloroform. Circular glass slides (18 mm diameter, .17 mm thickness) were coated by dipping the glass slide in the polymer solution and immediately drying it at room temperature. Poly(ethylene glycol) (PEG) substrates were prepared by photopolymerizing PEG diacrylate (MW: 1000)

on coverslips using photoinitiator with UV light. Polyurethane coated cover slips and PEG substrates were transferred to a 12-well plate, washed twice with Phosphate Buffered Saline (PBS) followed by washing twice with cell culture medium (CCM).

2.3. Polyurethane phase morphology

Phase morphology of PU substrates was analyzed using atomic force microscopy (AFM), transmission electron microscopy (TEM), Fourier-transform infra-red spectroscopy (FT-IR), and wide angle X-ray diffraction (WAXD). AFM imaging of the polymer coated glass slides was done using the dynamic force mode of a Park Systems XE-100 AFM. A silicon cantilever with a nominal spring constant of 40 N/m, resonant frequency of 300 kHz and tip radius of 10 nm was used. The phase shift angle (phase difference between the piezo driver signal and the oscillation of the cantilever as detected by the photodetector) of the dynamic force mode AFM is sensitive to tip-sample interaction. A smaller phase shift angle (i.e., darker contrast in the phase image) suggests a soft segment and a larger phase shift angle (brighter contrast) suggests a hard segment. For TEM, the samples were prepared by drop-casting a 1% (w/v) solution of the polymer in chloroform on a carbon coated copper grid (Tedpella, 400 mesh size) followed by evaporation of the solvent at room temperature. The samples were stained with a 2% solution of phosphotungstic acid before imaging. TEM images were obtained using a Jeol JEM-2010 TEM working at an operating voltage of 200 keV. FTIR spectra were recorded using a Bruker Vortex 70 spectrometer in the wave number range of 4000–400 cm^{-1} . The spectral resolution was 4 cm^{-1} and 128 scans were averaged. Absorbance ratio was calculated from the intensity of respective FTIR peaks. WAXD was done using Rigaku Ultima IV X-ray diffractometer and scanning was done from 5 to 60° at a rate of 0.5° per minute.

2.4. MSC morphology on PU substrate

PU coated coverslips (and control PEG substrates) were placed in a 12-well plate and approximately 3500 MSCs were added in each well for a relatively low cell density to avoid cell–cell contact. This allowed for assessing cell–matrix interactions without significant interference from cell–cell interactions. Prior to cellular morphology analysis cell viability, adhesion, and proliferation were assessed as described in SI. Cell morphology was assessed from brightfield images of MSCs captured with Nikon Ti-U Inverted Microscope equipped with camera at 10×. For a given sample, multiple images were acquired from randomly selected fields and representative images are presented from each group. Circularity index (CI) of cells was computed as, $CI = 4\pi A / P^2$, where A is the area of the cell and P is the perimeter of cells. Thus, CI value 1 indicates perfect circle and a value close to zero indicates a non-circular thin shape. Cell area and perimeter were calculated from the brightfield image using NIS element software. For a given surface, approximately 20 cells were examined from randomly selected fields to determine the cell surface area and circularity index. The experiments were performed in triplicate for a given surface and the experiments were repeated three times. A representative result from a given experiment is presented. Structural organization of MSCs on different substrates was observed through staining of F-actin and focal adhesion protein vinculin using Actin Cytoskeleton/Focal Adhesion Staining Kit according to the manufacturers' protocol. Briefly, MSCs were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, washed twice with wash

buffer (0.05% Tween-20 in PBS) and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Cells were washed twice with wash buffer followed by blocking with a 1% BSA solution in PBS for 30 min at room temperature. For focal adhesion staining, primary antibody (anti-vinculin) was diluted to a working concentration in the blocking solution (dilution 1:100) and incubated for 1 h at room temperature followed by washing with buffer three times at 15 min each. The goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody was diluted in PBS immediately before use and incubated for 60 min at room temperature. For actin staining, tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (dilution 1:200) was incubated simultaneously with the secondary antibody for 60 min at room temperature. It was then washed three times (15 min each) with the wash buffer. Following this washing step, cells were incubated with DAPI solution (dilution 1:1000) at room temperature for 2 min, followed by washing three times, 15 min each, with wash buffer. Fluorescence images of stained cells were examined on a fluorescence microscope (Ti-U Inverted Microscope, Nikon, Japan) equipped with appropriate channels at 20 $\times$. The cells were covered with PBS prior to visualization to prevent them from drying out.

2.5. PU gel and PU-MSC construct characterization

1 mL of PU suspension (formed during polymer precipitation) containing 1.7 ± 0.3 mg of polymer was transferred into a sterile 15 mL centrifuge tube and centrifuged at 3000 rpm for 5 min to form an aggregated PU gel. The supernatant was removed and the polymer was re-suspended in phosphate buffer saline (PBS) and centrifuged again to form the pellet. PBS was removed and this step was repeated twice with PBS and cell culture media (CCM). PU gels were characterized with scanning electron microscopy (SEM) and oscillatory rheology. SEM images were taken using a Hitachi S4000 with an operating voltage of 25 kV. Samples were coated with conducting carbon before imaging. To maintain the structure of the gels, PU gels were dehydrated by using graded ethanol steps followed by the addition of hexamethyldisilazane (Sigma, MO). Oscillatory rheology was performed using a Bohlin Instruments CVO Rheometer with 20 mm stainless-steel parallel plate geometry with a gap of 1.2 mm at room temperature (25 $^{\circ}$ C). Testing temperature was controlled using a CCE Temperature Cell with the novel Joule-Thomson Vortex cooling system. Small angle oscillatory shear measurements on the rheometer were conducted by frequency sweeps, varying the frequencies (0.1–10 Hz) with fixed strain (0.2%) which is in the linear viscoelastic region determined from amplitude strain sweeps conducted at a constant frequency of 1 Hz.

To entrap MSCs within PU dispersion, 1 mL cell culture media containing 1.5×10^5 MSCs were added to each centrifuge tube containing the polymer and mixed thoroughly using a pipette. The tubes were centrifuged as described to form a 3D construct of PU and MSCs. The supernatant medium was replaced with either CCM or chondrogenic differentiation media (CH) after 24 h depending on the experiment. PU-MSC constructs were placed in an incubator maintained at 37 $^{\circ}$ C and 5% CO₂ and media was changed on every third day. SEM of PU-MSC constructs were performed, as described earlier. The viability of MSCs entrapped in PU gels was correlated from the Alamar Blue assay. A series of constructs was prepared by varying the cell number from 0.8 to 2×10^6 and cells were allowed to stabilize

for 1 day. After 1 day 100 μ L of dye was added to each sample and they were incubated for 24 h. 100 μ L of the supernatant from each sample was withdrawn and its fluorescence intensity was measured with an excitation and emission wavelength of 570 and 590 nm, respectively. Fluorescence intensity observed from this assay directly correlates to the number of MSCs entrapped in the construct. Additionally, histological H&E staining was performed on PU-MSC construct to analyze cell size and distribution.

2.6. Chondrogenic differentiation of MSCs

Chondrogenic differentiation of MSCs entrapped in the PU was performed by treating PU-MSC constructs in a chemically defined chondrogenic media using STEMPRO® Chondrogenesis Differentiation Kit (Invitrogen, CA). As control, aggregated MSCs as cell pellet (containing same number of cells per pellet compared to PU-MSC constructs) were treated in chondrogenic media and PU-MSCs were treated in cell culture media (CCM). To define the significance of biphasic character of PU gels as chondrogenic matrix, MSCs were encapsulated in homogeneous monophasic PEG hydrogels formed by photocrosslinking of PEG-diacrylate (MW: 1000 to compare with PU with similar PEG soft segment) and treated in chondrogenic media. Briefly, 20 μ L of 10 wt.% solution of PEG diacrylate was mixed with 1.5×10^5 MSCs to a total volume of 40 μ L and photopolymerized with a photoinitiator at 365 nm for 10 min at an intensity of 8900 mW/cm². Chondrogenic differentiation of MSCs was induced for 21 days with changing media every 3 days and differentiation was assessed after 21 days for all the groups by examining chondrogenic markers.

2.7. Immunohistological and immunofluorescent staining

All the samples harvested at 21 days were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, washed thrice in PBS and infiltrated in agar. Histological sections of the constructs were obtained. GAG was stained with Alcian blue (at pH 10).

Immunohistochemical staining of collagen I and collagen II was performed with a commercial kit (Chondrex, VA) according to the manufacturers' protocol. Briefly, samples were sequentially treated with 2% bovine hyaluronidase (dissolved in PBS, pH 7.4), 1% H₂O₂ at 25 °C and blocking buffer with PBS washing in between steps at room temperature. Samples were incubated for 1 h with monoclonal antibodies to type I and type II collagen diluted at 1:1000 with dilution buffer followed by treatment with streptavidin peroxidase to localize primary antibodies and color was developed with 3,3'-diaminobenzidine (DAB) chromagen reagent. Immunofluorescent staining of aggrecan and cadherin-11 was performed after permeabilizing the cells with 0.1% Triton X-100 in PBS for 5 min at room temperature. Permeabilized cells were incubated with primary antibody (1:200 dilution) for 1 h at room temperature followed by 1 h incubation with either FITC or TRITC conjugated Goat anti-Mouse IgG secondary antibody (1:250 dilution). Samples were counterstained with DAPI to detect the nuclei. All the images were captured at 20 $\times$ with appropriate cameras fitted to Ti-U Inverted Microscope (Nikon, Japan).

2.8. Quantification of biochemical molecules

The total amount of GAG was quantified according to the established protocol by using a commercial kit from Astrate Biologics, VA. Quantification of GAG was performed after digesting the sample with papain. 1 mL of the digested solution and 1 mL of 1,9

dimethylmethylene blue dye were mixed in a test tube and absorbance of the solution was measured at 525 nm. Concentration of GAGs in the sample was determined by correlating the absorbance of the sample with that of a series of standard solutions of GAG made from chondroitin sulfate. The amount of GAG produced is normalized with the amount of DNA. Collagen II was quantified with an ELISA kit from Astrate Biologics, VA by following the manufacturer's protocol. Briefly, the samples were sequentially digested with pepsin and elastase followed by sequential incubation with biotin conjugated anti-collagen II antibody and avidin conjugated to Horseradish Peroxidase (HRP) secondary antibody. To this TMB substrate was added and the absorbance was measured to quantify the collagen content using a standard curve. The amount of collagen was normalized to DNA content. For both assays, DNA content was measured using a 'Quant-iT Picogreen' kit (Invitrogen). The constructs were digested using 1 mL 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA, 2 mM dithiothreitol and 300 µg papain at 60 °C for 24 h. 100 µL of the digested sample solution was used to estimate the amount of DNA using picogreen according to the manufacturer provided protocol. Aggrecan and cadherin-11 were quantified by measuring the fluorescence intensity using NIS element software from the fluorescent images and were normalized with respect to appropriate controls.

2.9. Statistical analysis

All experiments were done in triplicate. Data are presented as mean $\pm$ standard deviation of the mean. Data were analyzed by using a one-way ANOVA followed by the Tukey test for determining differences between groups. Differences were considered statistically significant with $p < 0.05$.

3. Results and discussion

3.1. Biphasic morphology of PEG based PUs

Polyurethane phase morphology is controlled by the segmental composition of polymers, namely the soft and hard segments. Intra and intermolecular interactions between PU segments create distinct biphasic morphologies at nanoscale dimensions. Two biodegradable PUs with PEG as soft segment were used to analyze the phase morphology. Hard segment of the PUs consisted of L-tyrosine based dipeptide, desaminotyrosine tyrosyl hexyl ester (DTH) as chain extender and either linear (HDI) or cyclic (HMDI) as aliphatic diisocyanate (Fig. 1). Depending on segmental interactions, PUs exhibit different degrees of phase structure which can be categorized as either phase separated or phase mixed. Both of these PUs are hydrophilic due to the presence of PEG as soft segment but their nanoscale morphology differs owing to the variation in hard segment. Fig. 2 shows the nanoscale morphology of two PUs; both AFM and TEM images show PEG-HMDI-DTH has relatively phase mixed morphology where the two segments are inter-mixed. In contrast, two phases in PEG-HDI-DTH are segregated at the nanoscale dimension with hard segment forming distinctly separate domains on soft segment matrix (darker regions in AFM are PEG soft segment and lighter regions are hard segment, whereas darker regions in TEM are hard segment and lighter regions are PEG soft segment). Since HMDI is bulky due to two cyclo-hexyl groups compared to linear HDI, hard segments are relatively less organized in PEG-HMDI-DTH due to steric hindrance. As a result, hard segments are diffused into soft segment leading to

phase-mixing. Whereas linear HDI creates well packed hard segment domains which are well segregated from the soft segment and are discretely present on the soft segment matrix. Alteration of PU nanophase morphology by changing the diisocyanate structure enables a distinct phase morphology which has been observed in similar studies [18]. These PEG based PUs are morphologically distinct at the nanoscale dimension although both are physicochemically hydrophilic. We further characterized this phase morphology by investigating the polymer structure with FT-IR and WAXD analysis (Fig. 3). In polyurethanes, H-bonds between the N–H and carbonyl functionality of urethane groups is one of the primary molecular interactions to induce biphasic morphology of PUs [10]. Increased H-bonding between urethane groups allows packing of the hard segment to form well segregated domains from the soft segment. Characteristically, both N–H and carbonyl stretching vibrations show two peaks corresponding to H-bonded and free state in the PUs (Fig. 3A). H-bonded stretching vibrations occur at lower frequency compared to free state (N–H stretching vibration in H-bonded and free state occurs at around 3300 and 3450 cm^{-1} respectively whereas carbonyl stretching vibration in H-bonded and free state occurs around 1700 and 1730 cm^{-1} respectively) [19,20]. In PEG-HDI-DTH, the peak intensity of H-bonded stretching of N–H and carbonyl is significant in comparison to their free state indicating that a major fraction of urethane groups are H-bonded in the hard segment. This enables phase-separation between soft and hard segment. In PEG-HMDI-DTH, bulky HMDI prevents H-bonding between the urethane groups of hard segments which is shown by relatively less intense H-bonded stretching vibrations of N–H and carbonyl. This results in phase-mixing in PEG-HMDI-DTH. Quantification of phase-mixing versus phase-separation character of PUs was made by calculating the absorption ratio of H-bonded to free peaks of N–H and carbonyl stretching vibrations (Fig. S1). For both N–H and carbonyl stretching, PEG-HDI-DTH has significantly higher ratios which show extensive inter-molecular H-bonding between urethane groups compared to PEG-HMDI-DTH. Thus, at the molecular level PEG-HDI-DTH exhibits phase-separation compared to phase-mixed morphology in PEG-HMDI-DTH. Further analysis of molecular level interactions is seen from WAXD (Fig. 3B). WAXD of PEG shows the characteristic diffraction pattern and pure DTH, a viscous liquid, shows a broad peak with moderate intensity at a 2θ value of 20.75° . DTH molecules can self-assemble by H-bonding and π – π interactions leading to small crystallites oriented in all directions which results in a broad peak [21–23]. Both PUs show similar diffraction patterns but with different intensities. There are no peaks corresponding to PEG but a broad peak corresponding to DTH along with small periodically repeating peaks are seen for both PUs. Since self-assembly of DTH prevents lamellar organization of PEG, PEG soft segment is amorphous in both PUs. However, the presence of a periodic diffraction pattern indicates that DTH undergoes self-assembly and maintains the partially ordered arrangement in PUs comparable to tyrosine based gels [24]. Variation in intensity of peaks is due to the population and distribution of small crystallites formed by self-assembly. In PEG-HDI-DTH, the hard segments are self-assembled into discrete crystallites separated by PEG. The crystallites are a few nanometers in size, well separated from each other and few in number, whereas in PEG-HMDI-DTH, there are more crystallites which are much smaller in size and are widely distributed. This is because the bulky HMDI in the hard segment perturbs the self-assembled phase structure. Since intensity of peaks in X-ray diffraction depends on the population of diffraction sites, PEG-HMDI-DTH shows peaks with higher intensity. These

results, both from FT-IR and WAXD, show the molecular level interactions inducing phase-separation in PEG-HDI-DTH compared to phase-mixing in PEG-HMDI-DTH. Since these morphological features are at the nanoscale dimension, we expect that cells will sense these features as cell–matrix interactions occur at the nanoscale dimension.

Our results show that structural variations in PU composition through changing diisocyanate structure can alter the phase morphology which is potentially a tool to regulate cell–matrix interactions and cell-fate. H-bonding interactions between segments, primarily through urethane linkages, and aromatic π – π interactions in DTH drive the self-assembly of PU segments, i.e. soft and hard segments, into distinct phases. These phases are nanostructured and, therefore, can interact with cells at a scale-length comparable to that occurring in natural matrix. Compared to PU phases, control of nanoscale cell–matrix interactions are primarily regulated by nanoscale immobilization of cell adhesive ligands and by topographic design through nano-patterns using lithographic techniques [25,26]. But these approaches do not create a differential phase structure which is driven by molecular self-assembly as observed in natural extracellular matrix. Phase morphology of PUs is molecularly driven and, therefore, is a molecular engineering tool to control cell–matrix interactions.

3.2. Cell–matrix interaction between MSCs and PEG based PUs

Mesenchymal stem cells (MSCs) are influenced by nanoscale features of the extracellular matrix [27,28]. Segmental character and nanoscale phase morphology of PEG-PUs can influence MSCs to control their function and fate in regenerative processes. Particularly in hydrophilic PUs, domains formed by hard segments are relatively more cell-interactive due to their hydrophobicity and increased mechanical stiffness [29–31]. PEG is known as a non-adherent matrix for cells due to its protein-resistant character [32,33]. Thus, PEG soft segments in these PUs act as cell-repellant whereas the hard segments are cell-interactive due to their hydrophobicity and protein attractive character. Furthermore, hard segment domains are mechanically stiffer than amorphous soft segments and are preferred by cells, as cells tend to orient toward stiffer matrix sites [34]. However, depending on PU phase morphology, hard segment domains are morphologically different at nanoscale dimensions in these two PUs and thus can control MSCs by providing distinct signals. MSC functions on the PUs were examined by analyzing cell adhesion, proliferation and viability. Compared to tissue culture polystyrene (TCP) substrate, MSCs exhibited lower adhesion on PU matrix because PU matrices consist of PEG soft segment, whereas on non-adhesive homogeneous PEG substrates very few cells were adhered (Fig. S2A). Significantly higher number of cells on PU compared to PEG substrate indicates that hard segments of PUs induced cell adhesion. Between two PUs, relatively more cells adhered on PEGHMDI- DTH (compared to PEG-HDI-DTH) due to the dispersed hard segment domains which presented high density of cell-interactive sites for cells to adhere. The viability of adhered cells on PU surfaces was not affected indicating that PUs were not toxic to MSCs (Fig. S2C). MSCs proliferated on both PUs' surfaces but at a significantly slower rate compared to TCP due to the PEG segments in PU (Fig. S2B). In general, these assays showed that PU matrices were compatible with MSCs without inducing cytotoxic effect on viability.

Influence of PU phase morphology on MSCs was analyzed from the cell size and shape because these features are indicative of the adhesive interaction with the matrix (Fig. 4). To identify the effect of the matrix, cells were seeded at low density to avoid cell-cell contact and the overall morphologies of MSCs on different substrate are shown in Fig. 4A. MSCs on PU matrices remained less spread with a circular shape and relatively smaller in size compared to on TCP due to the cell-repellant character of PEG soft segments on PU matrices but in comparison to homogeneous PEG substrate (where cells remained completely spherical and smaller in size compared to PUs), cells on PUs exhibited more spread-out morphology and increased size (Fig. 4B and C). This indicates the role of PU hard segment domains which provided cell-interactive loci for MSCs; however, depending on domain morphology, cell-matrix interactions varied on PU substrates. At an earlier time point (24 h), cells remained circular but exhibited significantly different morphologies on the two PU matrices. In PEG-HMDI-DTH, MSCs formed long pseudopod-like extensions whereas on PEG-HDI-DTH surface, MSCs formed bleb-like protrusions. Since PEG-HMDI-DTH exhibits phase-mixing behavior, MSCs extend these pseudopods to search cell-interactive hard segment domains which are diffused into the soft segment. At a later time point (48 h), cells assume an elongated morphology on PEG-HMDI-DTH becoming less circular compared to the 24 h time point. Although elongated, cells were not as spread out as seen on TCP and cell size remained small. Phase-mixed morphology of PEG-HMDI-DTH causes MSCs to elongate for increased interaction with hard segments. Since hard segments are mixed with soft domains, cell interactive sites in phase mixed morphology remain hidden to cells and cells extend to search for these domains, similar to MSC response observed on hydrophobically modified graft copolymers [35]. However, this response is different from the MSC behavior observed on PU matrix where soft segments are composed of hydrophobic poly caprolactone (PCL) [13]. In a phase mixed PCL based PU, MSCs extend pseudopods in multiple directions, but in PEG-PU the cell-repellant soft segment inhibits cell extension in multiple directions in spite of phase mixed morphology. Compared to phase-mixed PEGHMDI- DTH, phase-separated hard segment domains in PEG-HDI-DTH allowed MSCs to interact locally with small protruded structures. As a result, cells remain circular in shape and smaller in size at an earlier time point although they tend to interact with the matrix through bleb structures. Blebbing of MSCs is known when cells try to adhere in a non-adherent environment [36]. In contrast, MSCs on phase-separated PCL- PUs (where soft segments were hydrophobic) were able to stretch out while interacting locally with segregated hard segments. Thus, in phase-separated PEG based PU, cells remained confined with a circular shape. At a later time point (48 h) MSCs in PEG-HDI-DTH remained relatively circular and smaller in size compared to MSCs on other surfaces but the small bleb-like protrusions started extending. This extension is due to the cells' tendency to increasingly interact with the substrate over time.

To further analyze the cell-matrix interactions on PU substrates, we examined the expression of F-actin cytoskeleton and vinculin, a representative focal adhesion protein of MSCs (Fig. 5). MSCs formed well-defined F-actin stress fibers and organized focal points as adhesion junctions on TCP which indicates effective cell adherence. Compared to TCP, MSCs on PU substrate exhibited diffused F-actin expression without any filamentous fiber formation. In this context, F-actin expression of MSCs on non-adhesive PEG substrate was

more diffused and significantly less compared to PUs. At the 24 h time point, cells lacked in strong adhesive interactions with PU substrates which inhibited F-actin fiber formation which is consistent with rounded cell morphology. However, at 48 h, cells on PEG-HMDI-DTH formed distinct F-actin fibers but on PEG-HDI-DTH F actin were mostly diffused as spotty patterns and some peripheral localization. Concomitant to F-actin, vinculin expression of MSCs mirrored F-actin expression. Cells formed distinct focal adhesions on TCP at all time points, but on PU substrates the expression varied with time. At earlier time point, MSCs showed diffused vinculin on PUs but gradually formed some focal adhesion points as cells increasingly interacted with the PU matrix. This is significant because MSCs on homogeneous PEG substrate exhibited gradual decrease in vinculin stain with time whereas in PUs vinculin gradually matured into focal adhesions. This indicates that vinculin is recruited at the F-actin sites when cells start interacting with cell-interactive hard segment domains of the PU matrix. Thus, phase characteristics of PEG-based PUs influence cell morphology which is relevant in the context of the chondrogenic differentiation potential of MSCs. Rounded morphology of MSCs is critical for chondrogenic differentiation [8,37]. Associated with circular morphology, chondrogenic MSCs display a dispersed distribution of F-actin with some peripheral localization and few focal adhesions [38–40]. Additionally, fully differentiated chondrocytes can retain their chondrogenic phenotype with minimal spreading characterized by a few focal adhesion points but with less organized F-actin distribution [41]. This indicates that MSCs require optimal adhesive interaction with the substrate for efficient chondrogenesis but complete adhesion leading to spread-out cells with stress fibers and focal adhesions can disrupt chondrogenic differentiation. In this context, PEG-based PUs are useful because non-adhesive PEG segments prevent cell spreading and cell-interactive hard segment domains allow MSCs to interact with the substrate to strike a balance between cell adhesion and cell spreading. Particularly, phase separated matrix of PEG-HDIDTH can provide this balance (compared to phase-mixed PEG-HMDIDTH) where MSCs retain circular shape and F-actin remains predominantly diffused with some peripheral localization and formation of few focal adhesions. Phase-separated hard segment domains allowed MSCs to interact locally without any appreciable cell spreading. Conversely, MSCs formed elongated shapes as the cells increasingly interacted with the phase-mixed hard segments in PEG-HMDI-DTH. Cell morphology and F-actin/vinculin expression of cells are correlated to cell–matrix interactions [35,42,43]; therefore, MSC organization on two PU surfaces indicated the different levels of cell–matrix interactions. These differences can be attributed to surface physicochemical properties as phase mixing of hard segments reduce hydrophilicity of PEG based PUs [44]. The physicomechanical character of PUs can also contribute to this response as matrix stiffness regulates MSC morphology [44,45]. Overall, cell responses on 2-D PU substrates indicate that PU phases influence MSC behavior and phase-separated PU with PEG represents an appropriate chondrogenic matrix for MSCs. Furthermore, absence of specific cell-adhesive sequences, e.g. RGD (Arg-Gly-Asp), in hard segments of PUs can promote chondrogenesis as cell-adhesive ligands enhance cell spreading, leading to osteogenic differentiation.

3.3. Gel structure of PEG based PUs

Since chondrogenesis of MSCs requires a 3-dimensional (3D) micro-environment, we characterized a 3-D gel formed with PEG-based PUs. Fig. 6 shows the physicomechanical

character of PEG-HMDI-DTH and PEG-HDI-DTH as gels formed following precipitation in aqueous media from the reaction mixture. Morphologically, PEG-HMDI-DTH forms a solid elastomeric structure with high water content but lacks any microstructures (Fig. 6A), whereas PEG-HDI-DTH forms a gel showing microporous structures (Fig. 6B) with agglomerated morphology following precipitation and centrifugation. This is due to fractal gelation of the polyurethane from the aqueous dispersion owing to aggregation and interconnection of dispersed PU phases [46]. The gel like structure in PEG-HDI-DTH is attributed to segmental morphology where non-covalent molecular interactions assemble hard segment domains as physical crosslinks between PEG segments. H-bonds between urethane linkages and aromatic interactions between tyrosine residues allow hard segments to form nanophasic structures which are morphologically separated from the soft segment and, thus, act as physical crosslinks. Physical crosslinks from hydrophobic nanodomains have been observed in polyurethane and non-polyurethane based polymeric materials [11,47–50]. In contrast, hard segments of PEG-HMDI-DTH cannot form well-defined physical crosslinks to produce the effective gel structure due to the absence of organized hard-segments. This feature is further verified from the rheological analysis of PEG-HMDI-DTH and PEG-HDIDTH gels in the linear viscoelastic region determined by amplitude sweep at a constant frequency of 1 Hz (Fig. S3). Oscillatory frequency sweep of PEG-PUs was performed to measure the temporal evolution of storage modulus (G'), loss modulus (G'') and phase angle (δ) which show distinctly different behavior for PEG-HMDI-DTH (Fig. 6C) and PEG-HDI-DTH (Fig. 6D) indicating the difference in their gel structure. PEG-HMDI-DTH exhibits an increase in storage modulus (G') and loss modulus (G'') with increasing frequency which indicates entanglement of PU segments forming interpenetrated structures due to phase-mixing. At low frequency, high relaxation time provides entangled segments of PEG-HMDI-DTH to relax but gradually become more elastic ($G' \gg G''$) at higher frequencies when relaxation time is smaller compared to segmental relaxation. This type of frequency dependent response characterizes intermolecular chain entanglements as observed in associative polymers and gels [51–53]. Whereas PEG-HDI-DTH exhibits nearly frequency independent G' (which is greater than G'') over the tested frequency range; indicating dominant elastic character. However, at higher frequency viscous modulus (G'') shows frequency dependence indicating the reduced relaxation of PEG chains associated with water molecules. This behavior is indicative of viscoelastic relaxation modes corresponding to segmental composition and shows a relatively high degree of intermolecular junctions as observed in self-assembled gels [48,54–57]. The stable and well-organized gel structure of PEG-HDI-DTH is attributed to segregated hard segments acting as physical crosslinks between the water embedded PEG soft segments. It is important to recognize that PU gel structures are dependent on polymer composition, solid content, temperature, solvent and processing conditions [58,59]; however complete characterization is beyond the scope of this research. Nevertheless, rheological characterization provides evidence for gel structure from PEG-PUs which is attributed to the segmental composition of PUs.

3.4. MSC based construct with PEG based PUs

Physicochemical characteristics and morphology of PEG-HDI-DTH gel enable efficient entrapment of cells during centrifugal aggregation, whereas precipitation of PEG-HMDI-

DTH as solid elastomeric mass prevents cell entrapment. Macroscopically, MSCs entrapped in PEG-HDIDTH gel appear semi-solid in structure (Fig. 7A) and cells were effectively entrapped within the gels and remained circular in shape as seen from histological analysis (Fig. 7B). Histological staining of the MSC-gel construct showed a high density of rounded MSCs in the gel and this was further confirmed by the SEM image (Fig. 7C). Moreover, the viability of entrapped MSCs was measured with different concentrations of cells which showed that MSCs were viable within the gels and the viability signal was proportionate to the cell density (Fig. 7D). These results suggest that PEG-based PUs from PEG-HDI-DTH can form 3-D cell-matrix constructs for MSCs. MSC based PEG-HDI-DTH gel constructs were further characterized from rheological analysis. An amplitude sweep of cell-laden PU gel at 1 Hz (Fig. 7E) shows a dominant elastic character with elastic moduli (G') greater than viscous moduli (G''). Frequency sweep (Fig. 7F) of the cell-laden gel at a constant strain of 0.2% shows that cell based gels remained predominantly elastic over the tested frequency ($G' \gg G''$) but showed weak frequency dependent behavior at higher frequencies. Compared to cell-free PU gels (see Fig. 6D for PEGHDI- DTH gel), both the moduli of cell-laden gels increased which is likely due to the interaction between the PU matrix through hydrophobic domains. A similar increase in dynamic moduli has been observed between cells and hydrophobically modified alginate gels which is attributed to the interactions of cells with hydrophobic domains [60]. Furthermore, G'' values show fluctuations from the intermediate frequency range which are likely due to reduced relaxation and reorganization of PEG chains in the presence of cells, similar to responses in worm-like micelles and actin-based gels which cannot recombine to form original structures at high frequencies [61,62]. Taken together, these characterizations indicate that MSCs embedded in PEG-HDI-DTH form stable gels where physical crosslinks induced by the hard segment domains enable MSCs to be entrapped within the 3D matrix.

3.5. Chondrogenic differentiation of MSC in 3-D PEG based PU gel

Applicability of a PEG-HDI-DTH gel, with its biphasic morphology and high water content, as a chondrogenic matrix for MSC differentiation was examined after inducing differentiation for a 21 day period in chemically defined chondrogenic media. Chondrogenic differentiation of MSCs within PU gels was compared to that of MSCs maintained as a multicellular aggregation or as a pellet culture in the same chondrogenic medium, and to that of MSCs embedded in PU gels in the basal media without chondrogenic supplements. Fig. 8 shows immunohistochemical staining of glycosaminoglycan (GAG), collagen II, and immunofluorescent staining of aggrecan as expression of cartilage specific extracellular matrix molecules due to chondrogenesis of MSCs. MSCs in PU gels have a significantly higher expression of these chondrogenic markers, as seen from the positive stains, compared to MSCs in pellet cultures in chondrogenic medium. This suggests that the PU matrix provided a supportive role to differentiate MSCs into the chondrogenic lineage by providing guidance to synthesize chondrogenic matrix and subsequently retain the matrix molecules. To specifically define the effect of the PU matrix, MSCs embedded in PU gels were treated with basal media by excluding the chondrogenic stimulators. Qualitative expression of chondrogenic markers by MSCs under this condition was less compared to that of MSCs in chondrogenic media. However, even in the absence of chondrogenic supplements MSCs in PU gels showed relatively similar expressions of chondrogenic markers compared to MSCs

in the pellet culture treated with chondrogenic media. This suggests that the biphasic PU gels alone can induce chondrogenic fate in MSCs in the absence of soluble chemical stimulators, and the PU gel and chondrogenic media together have a synergistic effect on inducing chondrogenic differentiation of MSCs. Furthermore, Fig. 8 shows that MSCs under all conditions did not upregulate collagen I which is consistent with chondrogenic differentiation of MSCs and indicates that MSCs were not differentiated toward fibrocartilage or osteogenic lineage [63–65].

To further analyze the effect of a biphasic PU matrix on chondrogenic differentiation of MSCs, we quantified the expression of chondrogenic markers by MSCs in PU gels in chondrogenic media (PU + CH). Expression of chondrogenic markers by MSCs under this condition was compared to that of MSCs cultured as a cellular aggregate in chondrogenic media (Cell Pellet+ CH) and MSCs embedded in a PU gel but in basal media (PU+CM). In addition to these two controls, one additional control was included where MSCs were encapsulated within photopolymerized polyethylene glycol (PEG) and treated in chondrogenic media (PEG + CH). Photopolymerized PEG gels are monophasic in character due to chemically crosslinked PEG chains compared to biphasic PU gels although both matrices are essentially hydrogels in physicochemical nature. Therefore, comparison of chondrogenic differentiation of MSCs between PU + CH and PEG+CH groups categorically defined the role of the biphasic matrix in inducing chondrogenic differentiation. Solid content in both the gels was comparable and cell to polymer ratio was kept constant. Fig. 9A shows significantly low DNA content in PEG + CH group compared to other groups. This suggests that cell survival during the differentiation period is significantly less in PEG gel, similar to other studies [66], whereas the PU gel favored cellular survival (but no cell proliferation was observed as the DNA content remained relatively constant in the PU gel throughout the differentiation period, data not shown). However, no significant difference in DNA content was observed in PU gels (either in chondrogenic or basal media) compared to cellular aggregates which shows that PU gel has no inhibitory effect on cellular functions. Biosynthesis and accumulation of glycosaminoglycans (GAGs), as chondrogenic matrix, was detected from the biochemical assay which shows that MSCs in gels (both in PU and PEG gels) were able to produce larger amounts of GAG compared to cellular aggregates in chondrogenic media (Fig. 9B). This, in essence, shows the importance of the matrix for retention of the chondrogenic matrix during differentiation. However, PU gels appear to be more effective in GAG production compared to PEG gels which indicates the stimulatory effect of the biphasic character of PU gels. Most significantly GAG production by MSCs in PU gels in the absence of chondrogenic stimulators was higher than that of MSCs in cellular aggregates in chondrogenic media. Similar to GAG, collagen II, a distinguishing chondrogenic matrix molecule, was significantly upregulated in PU gels compared to other controls (Fig. 9C), similar to the GAG expression pattern. Expression of collagen II in PU gels was more than 2-fold higher than that in PEG gels due to hard segment domains which retained the synthesized collagen within the matrix. While the increase in GAG content in PU gels (compared to that in PEG gels) was relatively smaller, although significant ($p < 0.05$), increase in collagen II was relatively higher in PU gels. Thus, biphasic morphology of PU gels specifically enhanced collagen II accumulation likely through hard segment domains which were able to interact with collagen due to hydrophobic and protein retention

characteristics. Similarly, collagen II expression in PU gels in the absence of chondrogenic media was significantly higher than that of cellular aggregates in chondrogenic media which shows that PU gels alone can stimulate collagen II synthesis. In general, enhanced expression of chondrogenic ECM through GAG and collagen II suggest the effectiveness of PU gels as a chondrogenic matrix. In addition to GAG and collagen II, expression of aggrecan, large aggregating proteoglycan, a major ECM component in hyaline cartilage [67], was quantified from the immunofluorescence. MSCs in PU gels expressed significantly more aggrecan than all the controls (Fig. 9D) including PEG gels. Collectively, this quantified analysis suggests that PU gels act as chondro-inductive matrices to MSCs with enhanced expression of chondrogenic ECM molecules. Biphasic PUs with distinct morphology stimulate MSCs into the chondrogenic lineage where the cells are rounded and are able to induce balanced interaction with the matrix to synthesize chondrogenic matrix molecules.

These results suggest the unique role of biphasic PUs, particularly in comparison to monophasic PEG, in providing matrix signals to MSCs for chondrogenic differentiation. To further analyze this effect of cell–matrix interaction on MSCs in PU gels, we hypothesized that the hard segment domain of the PU matrix provided distinctive interactive loci for MSCs to interact and initiate cell–cell aggregation within the matrix because the soft segment PEG acts as a cell-repellant domain. Cellular condensation is an important prerequisite step for chondrogenesis of MSCs, particularly during developmental stages [68–70], and the biphasic morphology of the PU provided a unique matrix to initiate mesenchymal condensation. Since cadherin-11 is one of the representative cell–cell adhesion molecules to promote chondrogenic differentiation [64,71], we examined cadherin-11 expression by MSCs in different conditions. Fig. 10A shows MSCs in a PU gel in chondrogenic media expressed significantly higher cadherin-11 which can be seen from localization of fluorescence around the cells compared to other controls. Quantified analysis shows (Fig. 10B) significantly ($p < 0.05$) higher cadherin-11 in MSCs in PU gels compared to PEG gels in chondrogenic media. This proves our hypothesis that MSCs in PUs undergo condensation and form cell clusters, while cells in PEG gels are relatively dispersed. MSCs require cell-interactive domains to condense during chondrogenic differentiation as seen in artificial matrices immobilized with chondroitin sulfate or N-cadherin which enhanced chondrogenesis of MSCs likely due to mesenchymal condensation [64,72]. In PU gels, a similar effect is provided by the hard segment domains which create cell-interactive loci for MSCs to condense and subsequently induce chondrogenic differentiation, comparable to hard-segment induced cellular aggregation in hybrid PEG gels [73]. Interestingly, cellular aggregation of MSCs (in chondrogenic media) did not over-express cadherin-11 although cell–cell contact was present [74]. In comparison, MSCs in PU gels in basal media showed relatively more cadherin-11 which further confirms the inductive role of the PU matrix to initiate cell–cell contact even in the absence of chondrogenic stimulators. Clearly, MSCs were able to receive similar signals from the cell interactive hard segment domains of the biphasic PU matrix whereas MSCs were not able to interact efficiently in pellets and in traditional PEG gels where secondary locations were absent. This shows matrix mediated cell–cell interaction induced through PU gels is crucial for chondrogenesis of MSCs. Expression of chondrogenic matrix molecules, i.e. collagen II, GAGs, and proteoglycan

corroborated cadherin-11 expression to substantiate the role of the biphasic domains in PU gels as an effective signal for chondrogenic differentiation.

Overall, this data shows the effectiveness and applicability of PU gels as a chondrogenic matrix for differentiating stem cells. Biphasic morphology due to the segmental structure of PUs enhanced MSC chondrogenesis indicating the importance of a multi-phasic matrix which can orchestrate matrix signals. In this study, PEG based PUs provided distinct signals to MSCs where PEG soft segments were cell-repellant but the hard segment domains were cell-interactive. Morphological organization of these phases balanced the cell–matrix interaction between MSCs and the PU gel to regulate chondrogenesis. Efforts to present secondary sites for cellular interactions in homogeneous PEG gels have been attempted by functionalizing PEG molecules with specific functional groups. For example, immobilization of cell adhesive RGD peptides in PEG promotes chondrogenesis of MSCs through cell adhesion or presentation of chondroitin sulfate in PEG hydrogels induces specific cellular interactions leading to enhanced chondrogenic differentiation [64,75]. In this context PU gels are unique because the hard segments can assemble into domains to create a biphasic morphology at nanoscale dimensions. Furthermore, these hard segment domains act as crosslinks through multiple physical interactions which eliminates the need for chemical crosslinks to stabilize the PU gel structure. Thus, PU gels due to their unique morphology serve a dual purpose by providing biphasic morphology and stabilizing gel architecture without chemical crosslinks which effectively enhance chondrogenic differentiation of MSCs.

4. Conclusions

In this study, we have defined the role of the phase morphology of PU matrices in controlling cellular functions which can regulate chondrogenesis of MSCs. Specifically; PU matrices designed from PEG soft segment exhibited distinct nanostructured phase morphology due to physical interactions between the segments. Interaction of MSCs with these PU matrices controlled cell shape and size with distinct cytoskeletal (F-actin) and focal adhesion (vinculin) expression. Phase-separated PUs induced MSCs in circular shape with dispersed F-actin and minimal focal adhesion expression. This indicates that phase-separated PUs can control MSC morphology by balancing cell–matrix interactions where phase-separated hard segment domains allowed MSCs to interact with the matrix and the PEG soft segment prevented cell spreading. To transform this 2-dimensional effect into 3-dimensional structure, PU gels were constructed from phase-separated PUs which formed a stable solid elastic gel structure with high water content. MSCs entrapped within the gels were viable and effectively differentiated into the chondrogenic lineage as examined by the expression of cartilage specific ECM molecules i.e. collagen II, GAGs, and aggrecan. Upregulation of chondrogenesis in PU gels was due to enhanced cell–cell interaction as evidenced by higher expression of cadherin-11 in PU gels. Hard segment domains of PEG based PUs enabled cells to interact with the matrix and PEG soft segments prevented cell spreading. These features along with the ability to form gel architecture promoted chondrogenesis of MSCs in PU gels. In the future, this study will focus on underlining the functional role of PU biphasic morphology in terms of physiochemical and mechanical characteristics to guide cell and characterize the temporal and spatial evolution of chondrogenic matrix molecules, including

chondrogenic gene expression. The properties of hydrophilic PU matrices represent a viable chondrogenic matrix which has immense potential for cartilage regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Segmental structure of polyethylene glycol (PEG) based polyurethanes designed with HDI (hexamethylene diisocyanate) or HMDI (4,4'-methylenebis(cyclohexyl isocyanate)) and desaminotyrosine tyrosyl hexyl ester (DTH). PEG-HDI-DTH forms phase-separated morphology whereas PEG-HMDI-DTH forms phase-mixed morphology.

Fig. 2.

Phasemorphology of PEG-PUs. AFM images shows soft (dark region) and hard (light region) segments are mixed together in PEG-HMDI-DTH but segregated into distinct domains in PEG-HDI-DTH (inset shows nanoscale organization of phases scale bar: 200 nm). Scale bar: 2 μ m. TEM images showing phase-mixing on PEG-HMDI-DTH and phase-separation in PEG-HDI-DTH (lighter regions are soft segment and darker regions are hard segment in TEM). Scale bar: 250 nm.

Fig. 3. Molecular interactions between PU segments. A) FTIR spectra show H-bonded and free N–H and urethane carbonyl (C=O) stretching which indicates differential molecular interactions leading to PU biphasic morphology. B) WAXD pattern of PEG-PU shows absence of PEG peaks, appearance of DTH halo, and sharp peaks at higher angle due to segmental interactions.

Fig. 4.

MSC shape and size are regulated by phase-morphology of PEG-PU. A) Representative brightfield images, B) circularity index and C) surface area of MSCs on PUs compared to TCP and PEG substrate.

Fig. 5.

Cytoskeletal organization (F-actin) and focal adhesion (vinculin) expression by MSCs on PU substrates compared to TCP and PEG substrate. (Scale bar: 50 μ m for both 24 h and 48 h images.)

Fig. 6.

Morphology and rheology of 3-dimensional PEG-PU gel. SEM morphology of PU gel. A) PEG-HMDI-DTH shows no microstructure and B) PEG-HDI-DTH shows aggregated gel with microstructure. Rheological analysis of PU gels from frequency sweep at constant strain (0.2%) for C) PEG-HMDI-DTH and D) PEG-HDI-DTH gels. (Scale bar: 10 μ m.) Both analyses were performed following extraction of PU from reaction mixture in aqueous media where PEG-HMDI-DTH formed solid elastomeric mass and PEG-HDI-DTH was centrifuged to form aggregated gel.

Fig. 7.

MSC-PU construct from PEG-PU gel with PEG-HDI-DTH. A) Formation PU-MSC construct by entrapping of MSCs in PU gels from PEG-HDI-DTH. B) H&E stained sections of PU-MSC constructs shows well distributed rounded MSCs within the construct. C) Pseudo-colored SEM image of MSCs (arrow) entrapped in PU gel. D) MSCs are effectively entrapped in PU gel where cell viability correlates to MSC numbers in the construct. E) Constant frequency (1 Hz) strain amplitude sweep of PU-MSC construct. F) Constant strain (0.2%) frequency sweep of PU-MSC construct in the linear viscoelastic region.

Fig. 8.

Immunohistological (for GAGs, Collagen II and Collagen I) and immunofluorescent staining (green fluorescence from aggrecan with DAPI stained nuclei in blue) of biomolecules expression by MSCs after chondrogenic differentiation shows effective chondrogenesis of MSCs in PU gel.

Fig. 9.

Quantified expression of biomolecules by MSCs after 21 days of chondrogenic differentiation. A) Amount of DNA synthesized by MSCs in PU gel both in chondrogenic and cell culture media was similar to that by MSCs in cell pellet but significantly higher than that in PEG gels. B) Total GAG, C) Type II collagen and D) Aggrecan expressions by MSCs were significantly higher in PU gels compared to PEG gels and cell pellet in chondrogenic media.

Fig. 10.

Cell–cell adhesion between MSCs in PU gels induces chondrogenic differentiation. A) Immunofluorescent staining of cadherin-11 (red fluorescence from cadherin with DAPI stained nuclei in blue) shows enhanced expression cell–cell adhesion molecule by MSCs in PU gel compared to controls. B) Cadherin-11 expression was significantly higher in MSCs in PU gel compared to PEG gel and cell pellet in chondrogenic media.