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Cell–material interactions on biphasic polyurethane matrix

Patrick Dicesare^{1,*}, Wade M. Fox², Michael J. Hill², G. Rajesh Krishnan², Shuying Yang³, and Debanjan Sarkar^{1,2}

¹Department of Chemical and Biological Engineering, University at Buffalo, The State University of New York, Buffalo, New York 14260

²Department of Biomedical Engineering, University at Buffalo, The State University of New York, Buffalo, New York 14260

³Department of Oral Biology, School of Dental Medicine, University at Buffalo, The State University of New York, Buffalo, New York 14214

Abstract

Cell–matrix interaction is a key regulator for controlling stem cell fate in regenerative tissue engineering. These interactions are induced and controlled by the nanoscale features of extracellular matrix and are mimicked on synthetic matrices to control cell structure and functions. Recent studies have shown that nanostructured matrices can modulate stem cell behavior and exert specific role in tissue regeneration. In this study, we have demonstrated that nanostructured phase morphology of synthetic matrix can control adhesion, proliferation, organization and migration of human mesenchymal stem cells (MSCs). Nanostructured biodegradable polyurethanes (PU) with segmental composition exhibit biphasic morphology at nanoscale dimensions and can control cellular features of MSCs. Biodegradable PU with polyester soft segment and hard segment composed of aliphatic diisocyanates and dipeptide chain extender were designed to examine the effect polyurethane phase morphology. By altering the polyurethane composition, morphological architecture of PU was modulated and its effect was examined on MSC. Results show that MSCs can sense the nanoscale morphology of biphasic polyurethane matrix to exhibit distinct cellular features and, thus, signifies the relevance of matrix phase morphology. The role of nanostructured phases of a synthetic matrix in controlling cell–matrix interaction provides important insights for regulation of cell behavior on synthetic matrix and, therefore, is an important tool for engineering tissue regeneration.

Keywords

mesenchymal stem cell; polyurethane; cell–matrix interaction; cellular organization

INTRODUCTION

Biomaterial-based regenerative tissue engineering with stem cells offers a viable treatment strategy for defective and dysfunctional tissues and organ. The essential role of biomaterial is to provide the biochemical and biophysical milieu which can present orchestrated signals to cell. As a temporary substitute of natural extracellular matrix, biomaterial-based artificial matrix should biomolecularly interact with cells (or stem cells) with spatial and temporal control to guide cellular functions for tissue formation. Thus, control of cell–matrix

interactions is important for effective and functional tissue regeneration. Current efforts have, therefore, been focused on designing biomaterials which can mimic the cellular microenvironment to converge the cues in proper signaling cascades.¹⁻³

Biodegradable polyurethanes (PU) are designed and utilized as synthetic biomaterials for tissue engineering applications which show the usefulness of PU as synthetic matrix.⁴⁻⁶ Structurally PUs are segmental due to the presence of “soft” and “hard” segments which creates a biphasic characteristics of PU matrix.^{7,8} Distinct physicochemical characteristics of PU segments induce intersegmental and intrasegmental interactions which create biphasic morphology with nanostructured phase domains.⁹⁻¹¹ Depending on the molecular structure and composition, the segmental interactions of PUs lead to different phase morphologies which can be either “phase-separated” or “phase-mixed.”⁸ Phase-separated morphology results when the interactions are dominant within the same segments whereas phase-mixed morphology occurs when interactions between the different segments are prevalent. Most importantly, the size, shape and orientation of nano-structured phase domains of PUs can be controlled by engineering the polymer structure and composition. Studies have demonstrated that PU domain structure can be modulated into spherical, cylindrical, or fibrillar structure with defined interdomain spacing.^{12,13}

Although degradable PUs have been used as tissue engineering matrices and scaffolds, the influence of PU phases on cellular functions and processes has not been examined. This is particularly significant because macromolecules, for example, proteins, proteoglycans, in natural extracellular matrix are present in particular arrangement with nanoscale dimensions for specific cell–matrix interactions. For example, adhesion of cells to sequence of Arg-Gly-Asp (RGD) motif (present in proteins such as fibronectin) is dependent on the conformation, spacing (on the adhesion protein) and the activity of the RGD sequences which are present in specific fragments of fibronectin with a periodicity below 70 nm.^{14,15} Additionally, fibrillar organization of fibronectin clusters these adhesion sequences in nanoscale dimension.^{15,16} Thus, morphological features of matrix molecules at cell–matrix interface play a major role in dictating the cell to differentiate, proliferate, migrate, apoptose, or perform other specific functions in a coordinated fashion. Studies have shown the role of interfacial properties in controlling range of cellular functions using artificial extracellular matrices. Matrix characteristics e.g. hydrophobicity, rigidity, and nanoscale topography have influenced the adhesion, migration and differentiation of mesenchymal stem cells (MSCs).¹⁷⁻¹⁹ Studies with other cells have also implicated the role of matrix in controlling cellular functions.^{20,21} These results indicate that nanostructured phase morphology of PU matrices can influence cell by inducing specific interactions at nanoscale dimensions.

Thus, in this study, we addressed the role of PU phase structure and morphology in controlling function of human MSCs which are therapeutically relevant cells for tissue regeneration and cell-based therapies.²² Furthermore, MSCs are relevant in tissue engineering applications due to their ability to differentiate into multiple lineages and induce pro-angiogenic effect.²³⁻²⁵ We designed degradable polyester PUs with polycaprolactone (PCL) as soft segment with aliphatic diisocyanates and L-tyrosine-based dipeptide chain extender as hard segment. Structural variations in PU were introduced by altering the molecular weight of PCL and structure of the diisocyanate. By varying the structure of PUs, phase morphology of PU substrates were altered to show the role of phase morphology in controlling the adhesion, cytoskeletal organization and migration of MSCs.

MATERIALS AND METHODS

Polycaprolactone (PCL) with number average molecular weight of 530 and 1250 were purchased from Sigma Aldrich and Polyscience, respectively. All other chemicals and

solvents were purchased from Sigma Aldrich (MO) and were used as received unless otherwise noted. The cover-slips 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), PrestoBlue™ cell viability reagent, Live-Dead® assay and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen, CA. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, GA.

Polyurethane synthesis and substrate preparation

PU were synthesized according to established two-step poly-condensation reaction between PCL, aliphatic diisocyanates, and L-tyrosine-based dipeptide chain extender, desamino tyrosine tyrosyl hexyl ester (DTH) as shown in Scheme 1.²⁶ DTH was synthesized from hexyl ester of L-tyrosine and desaminotyrosine through carbodiimide coupling reaction and purified according to the established protocol.^{27,28} For polyurethane synthesis, PCL was reacted with diisocyanate at 1:2 molar ratio in DMF as solvent at 90°C for 5 h to form isocyanate terminated prepolymer. Prepolymer was then chain extended with DTH at a 1:1 molar ratio at 90°C for 12 h. After the completion of reaction, the reaction mixture was added drop wise to diethyl ether at room temperature to precipitate the polymer as a light yellow sticky solid. The precipitated polymer was filtered using sintered glass funnel, washed with copious amount of ether and dried under vacuum at room temperature for 24 h. Three PU were synthesized using different PCLs and diisocyanates as shown in Scheme 1 and the nomenclature of the PU and relative composition (derived from weight percentage of respective components from the theoretical values) is described in Table I. The resultant PU polymers are composed of alternating “soft” amorphous segments of diols, and “hard” segments of diisocyanate and chain extender sections and the structure of the PU are shown in Scheme 1. As polyurethane phase characteristics are controlled by the segmental structure and interactions, these PU allow examination of two structural effects: variation in soft segment molecular weight (between PCL530-HDI-DTH and PCL-HDI-DTH) and diisocyanate structure (between linear diisocyanate in PCL-HDI-DTH and bicyclic one in PCL-HMDI-DTH). The influence of these structural variations on the biphasic morphology will provide a guideline to regulate polyurethane phase morphology. Two-dimensional polyurethane substrates were prepared by spin-coating of 18 mm round glass-coverslips with 1 wt % polyurethane solution in chloroform and subsequent drying of coated coverslips at room temperature under vacuum for 24 h. Prior to any cell experiments, vacuum dried coverslips were sterilized under UV-light in laminar flow hood for 12 h followed by incubation with cell-culture media for 12 h. Coverslips were coated with three PU and PCL by this method. For all experiments, PCL coated surface was used as a control to the polyurethane surfaces to assess the role of polyurethane phase morphology.

Transmission Electron Microscopy (TEM)

The phase morphology of the polymers was observed on a Transmission Electron Microscope (Jeol JEM 2010 operating at 200 kV). 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 on the grids were stained using a 2% solution of phosphotungstic acid and dried at room temperature overnight before imaging.

Fourier Transform Infra-Red (FTIR) spectroscopy

1% (w/v) solution of the polymer was made by dissolving the 10 mg of the polymer in 1 mL of chloroform. Thin films of the polymers were cast by slow evaporation of the solvent at room temperature. The films were characterized using Bruker Vertex 70 FTIR spectrometer having a single bounce ATR accessory. Spectra of the samples were obtained at room

temperature averaging 100 scans in the wavenumber range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} . Representative spectra of the PU in wavenumber region 1600–1800 cm^{-1} are presented for evaluation of polyurethane phase characteristics.

MSC phenotype and morphology

MSCs were culture expanded in α -MEM, supplemented with 15% FBS, 1% (v/v) L-Glutamine and 1% (v/v) Penn-Strep, at 37°C and 5% CO_2 . All experiments were performed using MSCs at passage number 4–6.

Polyurethane and PCL-coated coverslips were placed in 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 assessing cell–matrix interactions without significant interference from cell–cell interactions. Cell viability and cell adhesion were assessed after 24 h of cell-plating on the surfaces. After 24 h, the supernatant media was aspirated (similar quantities of floating cells were observed between the groups) and washed with PBS to remove nonadhered and dead cells followed by cell viability measurement with PrestoBlue™ cell viability reagent and Live-Dead® assay according to manufacturer's protocol. Viability from PrestoBlue™ was expressed as percentage of viable cells by considering the viability of cells grown on tissue culture polystyrene as 100%. Fluorescent images from randomly selected spots were used to assess cell viability in the assay and representative images from the PU are presented. Cell adhesion was quantified by manually counting the cells from randomly selected field under the microscope at brightfield and 10× magnification. Cell proliferation was quantified; similarly, 24 h after cells were plated by manual counting from randomly selected field under the microscope at brightfield and 10× magnification over a period of 3 days.

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 field and representative images are presented from each group. Circularity index (CI) of cell was computed as, $\text{CI} = 4\pi A/P^2$, where A is the area of the cell and P is the perimeter of cells. Thus, CI value 1 indicates perfect circle and value close to zero indicates non-circular thin shape. Cell area and perimeter was calculated from the brightfield image using NIS element software. For a given surface, approximately 20 cells were examined from randomly selected field to determine the cell surface area and CI. The experiments were performed in triplicate for a given surface and the experiments were repeated for three times. A representative result from a given experiment is presented.

Cellular organization of MSCs

Structural organization of MSCs on different substrate was observed through staining of F-actin and focal adhesion protein vinculin using Actin Cytoskeleton/Focal Adhesion Staining Kit (Chemicon, Millipore, MA) according to manufacturers' protocol. Briefly, MSCs were fixed with 4% para-formaldehyde in PBS at room temperature for 15 min and washed twice with wash buffer (0.05% Tween-20 in PBS). Then the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature and washed twice with wash buffer followed by blocking with 1% BSA solution in PBS for 30 min at room temperature. For focal adhesion stain, 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-conjugated secondary antibody was diluted in PBS immediately before use and incubated for 60 min at room temperature. For actin stain, tetramethylrhodamine isothiocyanate-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 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. Expression of F-actin and vinculin was quantified by measuring fluorescence intensity using NIS element software from the fluorescent images and was normalized with respect to appropriate controls.

Migration of MSCs

Cell aggregates of MSCs were used to examine the migration of MSCs on polyurethane substrate. Cell aggregates were prepared using the hanging drop method by using approximately 10,000 cells per 30 μ L of drop. Aggregates were suspended over cell culture media and incubated for 24 h before seeding on the substrate in 12-well plates. Images were acquired over a period of 24 h at definite time intervals post seeding using a Nikon microscope and software at 4 $\times$ magnification. Measurements were taken from the edge of the aggregate to the furthest cell in the field-of-view in five randomly selected directions. Results are presented as average from three cell aggregates for a given surface. Experiments were performed on three replicates for a given surface and were repeated twice. Quantified results and migration images are presented from representative experiments.

Statistical analysis

Data from each experiment are presented as average as mentioned with standard deviations. Statistical analysis was performed by analysis of variance (ANOVA) with Tukey's test and statistical significance was considered at $p \leq 0.05$ (denoted *) and was considered nonsignificant at $p > 0.05$ (denoted "ns").

RESULTS

Phase morphology of PUs

PU are essentially biphasic due to the presence of soft and hard segments. In generic terms, biphasic morphology of PU can be categorized as phase-mixed and phase-separated depending on the organization of phases (Scheme 2).^{8,11} Phase-mixed morphology arises from intermixing of two segments due to structural and functional interactions and results in dispersion of one phase into the other. In contrast, phase separated morphology arises from segregation of phases when the interactions between the phases are low. Structural variations in the PU produced significant differences in morphological features and these biphasic morphologies of PU are characterized by TEM and IR studies. From TEM images (Fig. 1), the two phases are distinguished by the staining: darker regions represent hard segment (diisocyanate and DTH) and lighter regions represent soft segment (PCL). Figure 1(A) shows the uniform monophasic morphology of PCL which is the soft segment component in the PU. TEM images of the PU show the presence of light and dark regions indicating biphasic morphology. For PCL530-HDI-DTH [Fig. 1(B)], hard segment domains are higher than soft segment due to high hard segment content and continuous distribution in the matrix through some degree of intermixing with soft segment. In contrast, PCL-HDI-DTH as shown in Figure 1(C), displays distinct hard and soft segments with lower degree of intermixing compared to other polyurethane.²⁹ Orientation of hard segment domains indicates cylindrical or fibrillar arrangement of hard segments which are segregated from soft segments in PCL-HDI-DTH. Whereas, polyurethane with cyclic diisocyanate, that is, PCL-HMDI-DTH [Fig. 1(D)] shows hard segment regions with relatively higher degree of mixing with soft segments, that is, hard segment domains are more dispersed in soft segment and smaller amount of segregated hard segment domains are present compared to HDI based

PU.^{29–31} Additionally, the segregated portions of hard segment domains in PCL-HMDI-DTH are spherically oriented. Equally important is the dimension of the domains, specifically hard segment domains, which are in the nanoscale range.

Phase domain morphology of these PU was further verified with FTIR and the regions corresponding to carbonyl groups associated with ester, urethane and amide bonds are shown in Figure 2. All three PU show additional carbonyl peaks in the region 1720–1680 cm^{-1} corresponding to urethane carbonyl indicating the presence of hard segment domain. PCL530-HDI-DTH shows higher segment content from area under urethane carbonyl region compared to other two PU. Presence of two urethane carbonyls: Hydrogen-bonded (H-bonded) and free urethane carbonyl (H-bonding occurring between urethane N–H and carbonyl groups) are indicators of phase morphology with H-bonded urethane indicating phase-mixed behavior and free urethane indicating phase segregated behavior.^{32,33} FTIR spectra show all PU exhibit different degrees of phase mixing which is also observed from the TEM images. However, overlapping of different carbonyl peaks from multiple functional groups does not allow quantitative definition of the degree of phase-mixing and phase-segregation. In general, PCL530-HDI-DTH shows significant phase mixed behavior due to high hard segment content. PCL-HDI-DTH shows low phase mixing due ordered structure of diisocyanate and cyclic diisocyanate in PCL-HMDI-DTH induces high level of phase mixing due to less ordered structure. It is important to note, preliminary analysis with atomic force microscopy reveals no significant differences on surface topography on the polyurethane (and PCL) surfaces (results not shown). Additionally, the polyurethane surfaces (and PCL) exhibit similar hydrophobic character as indicated by their water contact angle analysis.^{34,35} Thus, the nanostructured phase morphology is a major determining difference which distinguishes these surfaces and can be attributed as a significant regulator for cell behavior.

MSC phenotype on polyurethane matrix

Viability of MSCs examined on all the polyurethane matrices indicates that cells are viable on the polyurethane substrate. Cell viability measured by PrestoBlue™ Cell Viability Reagent Assay was in the range of 75–85% for all the PU [Fig. 3(A)] and was comparable to cell viability measured on pure PCL which is known as biocompatible polymer. Additionally, cell viability was confirmed by Live-Dead® assay, as shown in Figure 3(B). This assay also confirms that most MSCs exhibit green fluorescence and remain viable on the polyurethane substrate. In combination, these assays indicate the nontoxic nature of the PU to MSCs.

Cell adhesion quantified by the number of cells adhered on polyurethane substrate shows that MSCs adhered to poly-urethane surface without any significant difference among the different groups [Fig. 4(A)]. By 24 h, MSCs adhered to all the polyurethane substrates and no differences were observed compared to PCL or TCP. This is significant because the presence of biphasic morphology on polyurethane surface did not affect the adhesion of MSCs. These results show that the number of MSCs adhered on polyurethane surface remains unchanged irrespective of cell adhesion mechanism.

Proliferation of the adhered cells on polyurethane matrix was observed over a period of 3 days and was compared to PCL and TCP surfaces [Fig. 4(B)]. MSCs proliferated on PCL-HDI-DTH and PCL-HMDI-DTH surface at a similar rate compared to TCP but were significantly higher compared to PCL surface at the end of 3 day period. Whereas, PCL530-HDI-DTH showed similar proliferation rate compared to PCL. This result shows presence of two phases in PU selectively plays a role in the proliferation of MSCs. It further indicates that polyurethane substrates have no toxic effect on the proliferation of MSCs.

MSC morphology on polyurethane matrix

Shape and size of MSCs adhered on polyurethane substrate was examined from brightfield images of cells on respective surfaces after 24 h [Fig. 5(A)]. Visual examination of cellular morphology on different surfaces indicated the importance of polyurethane phases in controlling cellular morphology. MSCs on pure PCL surface exhibited spindle shaped morphology with unidirectional extensions, a characteristic of MSCs. In PCL530-HDI-DTH surface, cells assumed more spread out morphology, indicating the role of hard segment domains. MSCs can sense the presence of higher content of hard segment domain (compared to soft segment domain) with continuous distribution which favored cell adhesion and spreading. The role of biphasic polyurethane morphology was evident from the other polyurethane surfaces. PCL-HDI-DTH is chemically similar to PCL530-HDI-DTH but hard segment content is lower and not distributed with a phase mixed morphology. With relatively low phase mixing, MSCs on PCL-HDI-DTH exhibits elongated morphology with unidirectional extended protrusions. Interestingly, this type of morphology is similar to MSC morphology on PCL surface but fibrillar arrangements of hard segments in PCL-HDI-DTH corroborate MSC morphology. In contrast, MSCs on PCL-HMDI-DTH showed more spread out morphology with pseudopod like extensions in multiple directions. A major part of the hard segments in PCL-HMDI-DTH is in phase-mixed morphology with few discrete hard segment domains which are spherical. MSCs sense this morphological attribute to spread out with multidirectional pseudopod extensions. Thus, the similarities and/or differences of MSC morphology observed on polyurethane surface are related to phase-morphology of polyurethane matrix. These morphological features are shown by measuring the cell surface area in Figure 5(B) and CI in Figure 5(C). Surface area and CI values from all the substrates shows the spreading of MSCs but indicated subtle differences in cell morphology influenced by the phase architecture of substrate. Surface area measurement shows MSCs assumed highest surface area on PCL matrix with wide distribution ($1600\text{--}23,000\ \mu\text{m}^2$; $9644.4 \pm 7652\ \mu\text{m}^2$) but lowest CI (0.17 ± 0.06) compared to other substrates. This indicates MSCs assumed elongated morphology with unidirectional extensions. Compared to PCL substrate, MSCs on both HDI-based PU, that is, PCL-HDI-DTH and PCL530-HDI-DTH have significantly lower surface area but higher CI value. This shows presence of hard segment in PU controls cell spreading by restricting size and elongation of MSCs. In terms of cell size, surface area of MSCs in PCL-HDI-DTH and PCL530-HDI-DTH was similar but CI values were different. MSCs on PCL530-HDI-DTH were relatively more circular (CI = ~ 0.3) compared to MSCs on PCL-HDI-DTH (CI = ~ 0.2). This indicates that phase segregated hard segment with fibrillar morphology induces the cells to align with unidirectional spreading in PCL-HDI-DTH (but to a lesser extent compared to PCL substrate) whereas high hard segment content present as continuous matrix in PCL530-HDI-DTH allowed the cells to spread on continuous hard segment domain. On PCL-HMDI-DTH substrate, which exhibits relatively phase-mixed morphology, surface area of MSCs is similar to surface area of MSCs on PCL (but with less distribution) and is significantly larger compared to both HDI-based polymers. On the other hand, MSCs are relatively less circular on PCL-HMDI-DTH comparable to MSCs on PCL530-HDI-DTH. This is due to formation of pseudopod like extensions in multiple directions by MSCs on PCL-HMDI-DTH substrates. Thus, phase structure and morphology of PU play an important role in controlling the morphology of MSCs.

Cellular organization of MSCs on polyurethane matrix

Influence of biphasic structure and morphology of polyurethane substrate on MSC organization is examined from cytoskeletal assembly (F-actin orientation) and adhesion complex formation (expression of vinculin as representative adhesion protein). MSCs exhibited actin expression on PCL and polyurethane substrate as shown from the fluorescent images of F-actin in Figure 6. Overall expression of F-actin and organization of actin as

stress fiber were influenced by the matrix characteristics. Actin expression and organization of MSCs on PCL530-HDI-DTH were similar compared to PCL. But in PCL-HDI-DTH and PCL-HMDI-DTH, F-actin expression was significantly higher compared to PCL. Additionally, these polyurethane substrates induced actin organization into stress fibers to a greater extent compared to PCL530-HDI-DTH and PCL. High content of hard segment in PCL530-HDI-DTH is present as a continuous domain which does not change the cytoskeleton assembly of MSCs on PCL530-HDI-DTH substrate. But lower hard segment content in PCL-HDI-DTH and PCL-HMDI-DTH (compared to PCL530-HDI-DTH) shows the presence of hard segment as a differential phase domain to influence cytoskeleton organization. Additionally, the influence of phase characteristics was observed on the orientation of actin. Actin of MSCs on PCL and PCL-HDI-DTH substrate showed relatively parallel alignment whereas on PCL530-HDI-DTH and PCL-HMDI-DTH showed randomly organized structure in multiple directions. F-actin orientation correlated to the cell morphology and pseudopod like extensions of MSCs which are attributed to phase morphology of the substrate. To further examine the cytoskeletal organization, expression of vinculin for focal adhesion and stress fiber formation was detected for MSCs on all the substrate as shown in Figure 6. MSCs show negligible vinculin expression on PCL substrate which indicates cytoskeletal organization is not mediated by clustering of focal adhesions. No significant increase in vinculin mediated focal adhesion formation was observed on PCL530-HDI-DTH substrate in spite of the presence of hard segment domain. MSCs on PCL-HDI-DTH and PCL-HMDI-DTH substrate showed a significantly higher number of focal adhesion formation. Focal adhesions were co-localized with actin fibers on these two polyurethane substrates indicating that F-actin expression and organization were mediated by focal adhesions. Ratio of actin to focal adhesion expression further supported the fact that cytoskeletal assembly of MSCs on PCL-HDI-DTH and PCL-HMDI-DTH substrate was focal adhesion mediated. Closer examination of vinculin recruited focal adhesion formation is shown in Figure 7. No significant vinculin expression was observed from MSCs on PCL surface. Although quantitatively nonsignificant (compared to MSCs on PCL surface), presence of hard segment induced vinculin expression for MSCs on PCL530-HDI-DTH substrate was observed. Vinculin recruited focal adhesions on MSCs were smaller in size (within the range 2–6 μm) and fewer in number. However, nonorganized expression of vinculin was observed from blurred imaging which indicates improper activation of adhesion proteins and incomplete focal adhesion formation leading to less stress fiber formation.³⁶ However, MSCs on PCL-HDI-DTH substrate formed larger and elongated focal adhesions ($> 20 \mu\text{m}$) which indicate the formation of matured focal adhesions.^{37,38} In addition, some nonorganized vinculin expression was also observed. Larger focal adhesions with parallel alignment match orientation of stress fibers and the morphology of hard segment domain in PCL-HDI-DTH. MSCs on PCL-HMDI-DTH substrate showed a distribution of focal adhesions. Both smaller and elongated focal adhesions were observed on this polyurethane substrate. This corresponds to the phase morphology of PCL-HMDI-DTH where considerable phase-mixing occurs. Thus, polyurethane phase structure and its morphology at nanoscale dimension control the cellular organization by influencing focal adhesion and actin skeleton organization.

Migration of MSCs on polyurethane substrate

Polyurethane phase morphology induces a significant effect on the migration of MSCs. Differences were observed in the movement of MSCs on the polyurethane substrate which corroborates to phase structure and morphology. Movement of MSCs on PCL-HMDI-DTH was highest compared to all other substrates which MSCs had a similar rate of migration as shown in Figure 8(A). Migration of MSCs on PCL-HMDI-DTH was consistent with the morphology of MSCs on this polyurethane substrate. MSCs traveled longer distance on PCL-HMDI-DTH substrate with multidirectional pseudopod like extensions by extending in

different direction in search of a hard segment domain which is phase mixed with soft segment. On PCL-HDI-DTH, MSCs traveled slower compared to the HMDI based polyurethane substrate. This primarily due to phase segregated morphology of PCL-HDI-DTH where hard segments domains are separated from soft segment and MSCs sense these segregated domains as sticking point. Thus, MSCs remain less motile on PCL-HDI-DTH substrate. MSCs on PCL530-HDI-DTH substrate remain less migratory due high hard segment content which is sensed as a homogeneous matrix by cells. Similarly, MSCs on homogeneous PCL substrate exhibited less migration. Figure 8(B) depicts the migration pattern of MSCs on respective surfaces and indicates this fact. However, for any given surface, the differences observed from the individual cells shows the randomness of cell migration which explains the heterogeneous character of polyurethane matrix due to nanostructured phases. As this study was based on population of cells, it is important to note that cell–cell interaction might play an important role in the migration of cells in addition to cell–matrix interaction.³⁹

DISCUSSIONS

Fate of cells, including stem cells, is regulated by the myriad of signals received from the extracellular environment including the matrix. Thus, for regenerative tissue engineering strategies it is important to design an extracellular matrix which can induce and control the stem cell by providing definitive cues in an orchestrated manner. Recent studies have demonstrated the cell–matrix interaction at nanoscale level and the importance of these nanoscale structures on synthetic matrices as a key regulator of cellular features. In this study, we have shown the effect of nanoscaled phase structure and morphology of synthetic polyurethane matrices in controlling stem cell structure and function.

Segmental composition of PU induces different biphasic morphology owing to the intersegmental and intrasegmental interactions of PU segments, namely soft and hard segment.^{8,40} Hard segment of PU are relatively crystalline, polar, and hydrophobic compared to soft segments and thus, provide favorable sites for interaction with proteins and cells, for example, adhesion.⁴¹ We used L-tyrosine-based PU as model biocompatible and biodegradable polyurethane to examine the effect of biphasic polyurethane morphology. These PU were designed with polyester based soft segment, PCL with aliphatic diisocyanates (HDI or HMDI) and L-tyro-sine-based dipeptide DTH as hard segment. Through variation in the polyurethane structures, different phase morphologies were achieved and characterized by TEM and FTIR. In general, morphology of polyester based PU is phase-mixed due to interactions involving ester functionality but the extent of phase mixing (and therefore degree of phase separation) is dependent on polyurethane segments.^{29,42} Such variations are manifested at the nanoscale dimension and therefore can significantly influence the cellular features through eliciting specific signaling events. PCL530-HDI-DTH has significantly higher hard segment content which is largely present as continuous phase at the nanoscale dimension and thus, is sensed as homogeneous matrix by cells. However, compared to PCL, presence of hard segment in PCL530-HDI-DTH should provide different signals to MSCs. Compared to PCL530-HDI-DTH; other two PU have lower hard segment content, and therefore, can be sensed by cells as differential matrix. From TEM and FTIR, we observe that PCL-HDI-DTH and PCL-HMDI-DTH presents a differential matrix but with significant morphological differences. Mostly phase separated morphology is observed in PCL-HDI-DTH compared to phase-mixed morphology in PCL-HMDI-DTH due to linear HDI versus cyclic HMDI structure. These types of phase morphologies have been observed with HDI and HMDI based PU due to molecular level interactions between different segments.^{29,31} FTIR and TEM characterizations of the L-tyrosine-based PU reveal the nanoscale phase structure and morphology which is consistent with other PU. Cellular structure and functions are strongly guided by nanoscale features,

for example, topographies, ligand clustering, patterning.^{18,36,43} Therefore, nano-structured phase morphology of PU is a key regulator for controlling cell fate.

PU, including L-tyrosine-based PU, are cytocompatible to different cell line and this study also indicates that polyurethane matrix is nontoxic to MSCs.⁴⁴ Nanostructured features of synthetic matrices influence cell adhesion and proliferation through different mechanistic pathways. Particularly, cell adhesion has shown strong dependence on matrix nanostructures, for example, presentation of RGD ligands with defined density and spatial distribution “turns on” clustered integrin mediated cell adhesion.^{36,43} However, hard segment domains of polyurethane matrix did not specifically enhance the number of MSCs adhered compared to homogeneous PCL substrate. This is due to the cell-adhesive characteristics of PCL which is probably not different from the cell-adhesive characteristics of hard segment domains of polyurethane matrix. Additionally, nanostructured hard segment domains examined in this study do not contain cell adhesive unit (e.g., RGD like sequence) to stimulate enhancement in cell adhesion.⁴⁵ Interestingly, reduction of cell adhesion due to matrix nanostructures has also been observed in different systems.⁴⁶ Unlike adhesion, proliferation of MSCs was enhanced on two polyurethane substrates where hard segment domain presented differential matrix. Although, the effect of matrix nanostructures on cellular proliferation is not evident, several studies have shown enhanced proliferation on nanostructured surface.^{47,48}

Cell spreading and morphology are strongly influenced by matrix nanostructures and topographies. In general, cells tend to align and orient along the nanofeatures of the matrix which leads to elongated cell structure with lesser surface area. These morphological characteristics of cells have been observed on matrices with nano-pits and groves, nano-gratings, nanofibers, and nano-clustered ligands.⁴⁹ Morphology of MSCs on biphasic polyurethane matrices with nanostructured phases is consistent with these observations. MSCs assumed elongated shape with small surface area on PU with hard segment domains. Orientation of MSCs is influenced by organization of hard segment domains because cells preferentially interact with hard segment compared to soft segment. These results establish the role of biphasic morphology of PU as a key regulator for MSCs and, in general, cell morphology. However, future studies are important to understand how geometry and spatial organization of polyurethane phases are critical in controlling cell morphology.

Organization of cells, including MSCs, on nanostructured substrate has been investigated with different systems.^{18,50,51} These studies show F-actin-based cytoskeleton arrangement and focal adhesion formation is dependent on substrate nano-features. MSCs on polyurethane substrate showed that F-actin orientation and vinculin recruited focal adhesion are dependent on the nanostructured biphasic morphology of PU. Focal adhesions are formed through clustering of integrin ligand on cell membrane and are mediated through integrin-extracellular matrix binding.^{52,53} Stabilization and growth of focal adhesions are coordinated through intracellular and extracellular forces which in turn controls the F-actin orientation.^{54,55} As F-actin and vinculin expression of MSCs on polyurethane matrices differ from monophasic homogeneous PCL matrix, it indicates the role of polyurethane phases in controlling the cellular organization of MSCs. Thus, MSCs can sense the nanoscaled phase morphology to initiate and transmit specific cue for ultimate control of stem cell fate.⁵⁶⁻⁵⁸ Focal adhesion formation and organization of F-actin into stress fibers along the orientation of hard segment domains of polyurethane substrate indicates the localization of cytoskeletal force which is counterbalanced by the matrix force induced through nanostructured hard segment phases.⁵⁹ This was evident from distinct expression of F-actin and vinculin in MSCs on polyurethane matrices. However, it is important to sense the hard segment as nanostructured phases and as differential (in comparison to homogeneous) matrix component. Because presence of hard segment as homogeneous

matrix, as seen in PCL530-HDI-DTH, does not elicit significant focal adhesion formation. It is also important to observe the nonorganized focal adhesion and F-actin expression (from blurred expression) indicating phase morphology which is either homogeneous or phase-mixed, and therefore, cannot be sensed as differential nanostructured biphasic matrix. This effect of cellular organization was also manifested by migration of MSCs on polyurethane substrate. MSCs migrated towards hard segment domains in a phase-mixed morphology but remained less motile on substrates where hard segments were segregated. This is in contrast to migration of endothelial cells on polyurethane substrates which showed enhanced migration on phase-separated matrix.⁶⁰ However, it is important to note that cell migration is a highly coordinated event and is dependent on the cell type.

Taken together, these results show polyurethane phase morphology in nanoscale dimension can specifically interact with stem cells to induce specific effect on cell structure and function. This study has demonstrated the effect of different nanostructures on cell fate and of nanostructured phase morphology on stem cell function. Stem cell structure and morphology is a key to control stem cell fate including its differentiation potential.^{58,61} Several studies have shown the cell shape, cytoskeletal rearrangement and intracellular tensions regulate stem cell differentiation, for example, circular MSCs tend for adipogenic differentiation while noncircular MSCs differentiate into osteogenic lineage.⁶¹ Therefore, biphasic PU can be developed as a cell-instructive nano-structured material for regenerative applications. However, from a mechanistic point of view it is important to understand the role of these nanostructured phases in terms of physicochemical effect (e.g., hydrophobicity, protein adsorption etc.) and/or physicomachanical effect (e.g., surface rigidity, roughness, etc.). Future studies are required to underline these relationships.

CONCLUSIONS

Cell–matrix interaction is important for controlling cell fate including that of stem cells. Stem cells interact with extracellular matrix at the nanoscale dimension to receive signals, and therefore, synthetic matrices should provide nanostructured features to influence stem cells. Polyurethane phase morphology is nanostructured which can be controlled by the segmental structure and compositions. Stem cell structure and functions have shown dependence on this nano-structured phase morphology through defined cellular organization. This study provides significant understanding in controlling cell–matrix interactions through controlled matrix nanostructures and offers a tool to guide stem cell behavior in a predefined fashion for regenerative tissue engineering applications.

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FIGURE 1.

Transmission electron microscopic (TEM) images of polymer phase morphology indicated by the presence of phases with lighter regions presenting soft segments and darker region presenting hard segments of PCL (A) and three PU, PCL530-HDI-DTH (B), PCL-HDI-DTH (C), and PCL-HMDI-DTH (D). Scale bar ~500 nm.

FIGURE 2.

FTIR spectra of PU for phase morphology assessed by urethane carbonyl peak distribution in three PU and PCL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

FIGURE 3.

Viability of MSCs on polyurethane substrate measured at 24 h: (A) Cell viability measured from Cell Prestoblu[®] assay and normalized with respect to cells in tissue culture polystyrene and (B) Representative fluorescent image of three PU from LIVE/DEAD assay with green cell indicating viable cells and red cells indicating dead cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

FIGURE 4.

Phenotype of MSCs on polyurethane substrate and tissue culture polystyrene, TCP and PCL: (A) Adhesion of MSCs measured after 24 h of seeding by the number of cells per unit area, and (B) Proliferation of MSCs over a 3 day period. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com)]

FIGURE 5.

Morphology of MSCs on three PU and PCL substrate: (A) Representative brightfield image of MSCs on a given surface 24 h after seeding (scale bar: $\sim 100 \mu\text{m}$), (B) Quantified measurement for surface area of cells with individual cell measurement, (C) CI measurement for cells with individual measurement. Bar represents average values in surface area and CI measurement. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

FIGURE 6.

Organization of MSCs. Representative images from immunofluorescent staining of F-actin (red) and focal adhesion protein (vinculin, green) for MSCs on three polyurethane surface and PCL. Quantified expression of F-actin and vinculin measured by fluorescence intensity and the ratio of actin to vinculin as a measure of colocalization of actin expression and focal adhesion formation (scale bar: ~50 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

FIGURE 7.

Focal adhesion formation by MSCs on polyurethane substrate measured by immunofluorescence of vinculin (green) (scale bar:~20 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

FIGURE 8.

A: Migration of MSCs. Displacement of cells from an aggregate measured on polyurethane and PCL substrate over a 24 h period. **B:** Representative cell migration image on PCL (a), PCL530-HDI-DTH (b), PCL-HDI-DTH (c), and PCL-HMDI-DTH (d) surface at 22 hour time point. Dotted line indicates the migrating front of the cells. (Scale bar: ~400 μm) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

SCHEME 1.

Chemical structure of polyurethane segments corresponding to soft and hard segment component of the polymer and chemical structure of the polyurethane used in the study with cells.

SCHEME 2.

Schematic presentation of polyurethane segmental structure and the corresponding biphasic morphologies: phase-mixed and phase separated owing to interactions between polyurethane segments.

TABLE I

Structure and Composition of Polyurethanes Used for this Study

Polyurethane	Soft Segment PCL Molecular Weight	Hard Segment ^a Diisocyanate Structure	Soft Segment Content (wt %)	Hard Segment Content (wt %)
PCL530-HDI-DTH	530	HDI	41.5	58.5
PCL-HDI-DTH	1250	HDI	62.6	37.4
PCL-HMDI-DTH	1250	HMDI	57.1	42.9

^aHard segment of all three polyurethanes contain DTH as chain extender.