Engineering Cellular Response Using Nanopatterned Bulk Metallic Glass

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ABSTRACT Nanopatterning of biomaterials is rapidly emerging as a tool to engineer cell function. Bulk metallic glasses (BMGs), a class of biocompatible materials, are uniquely suited to study nanopattern–cell interactions as they allow for versatile fabrication of nanopatterns through thermoplastic forming. Work presented here employs nanopatterned BMG substrates to explore detection of nanopattern feature sizes by various cell types, including cells that are associated with foreign body response, pathology, and tissue repair. Fibroblasts decreased in cell area as the nanopattern feature size increased, and fibroblasts could detect nanopatterns as small as 55 nm in size. Macrophages failed to detect nanopatterns of 150 nm or smaller in size, but responded to a feature size of 200 nm, resulting in larger and more elongated cell morphology. Endothelial cells responded to nanopatterns of 100 nm or larger in size by a significant decrease in cell size and elongation. On the basis of these observations, nondimensional analysis was employed to correlate cellular morphology and substrate nanotopography. Analysis of the molecular pathways that induce cytoskeletal remodeling, in conjunction with quantifying cell traction forces with nanoscale precision using a unique FIB-SEM technique, enabled the characterization of underlying biomechanical cues. Nanopatterns altered serum protein adsorption and effective substrate stiffness, leading to changes in focal adhesion density and compromised activation of Rho-A GTPase in fibroblasts. As a consequence, cells displayed restricted cell spreading and decreased collagen production. These observations suggest that topography on the nanoscale can be designed to engineer cellular responses to biomaterials.

KEYWORDS: bulk metallic glass · biomaterials · nanorods · cell–nanopattern interactions · mechanosensing · traction force measurement

Novel patterning technologies have expanded the fundamental understanding of cellular function at the nano level, a new length scale in cell biology. Nanopatterning can be incorporated into implant design to increase the function and longevity of biomaterials and devices by orchestrating cellular responses, including the foreign body response (FBR). Moreover, nanopatterned biomaterials provide a unique platform to assess cellular responses to topographical cues.

Although nanoscale topography has been exploited with polymers, ceramics, and metals to investigate cellular response, limitations exist with these major material classes. Surface engineering of materials for biomedical applications demands consideration of multiple factors, including manufacturability, targeted in vivo functionality, and biocompatibility. Presently, engineered materials have limitations to meet all of these requirements. For example, nanopatterned polymers represent a readily manufacturable and cost-effective tool that can provide fundamental understanding of nanopattern–cell interactions; however, the comparably low yield strength and elastic modulus limit the range of applicability as structural biomaterials. In contrast, silicon allows formation of intricate small-scale, high aspect ratio nanopatterned structures, but

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undesirable mechanical properties and lack of biocompatibility limit its use in biomedical applications. Metals and metallic alloys such as titanium and stainless steel alloys have a high strength and stiffness and can be used to provide structural support or replace hard tissues, yet intrinsic length scale restrictions imposed by the grain size of conventional metals pose a challenge to achieve nanoscale feature sizes.\textsuperscript{7,8} A continuing need exists for biomaterials having strength and stiffness comparable to metals with the processability akin to polymers.

Versatile chemistry and amorphous atomic structure of BMGs enable a range of compositions that combine processability, as quantified by glass forming ability, and biocompatibility.\textsuperscript{9,10} In addition, the mechanical properties of BMGs combine elasticity, strength, and ductility, particularly when used at the nanoscale.\textsuperscript{11–14} Moreover, the unique processability of BMGs enables thermoplastic forming (TPF) in a nonrestrictive environment to produce a broad range of novel nanopatterned structures.\textsuperscript{15}

Work presented here employs nanopatterned BMG substrates produced using TPF to explore detection of nanopattern feature sizes by various cell types. BMG nanorod arrays with feature sizes ranging from 55 to 200 nm were produced. Three cell types, namely, fibroblasts, macrophages, and endothelial cells were analyzed for nanopattern-induced cytoskeletal remodeling. Fibroblasts, which mediate fibrosis and encapsulation of biomaterials leading to implant failure, were found to detect the smallest nanopattern feature size examined in this study (55 nm). Primary macrophages are involved in the inflammatory response to implants and release reactive oxygen species and degradative enzymes. These cells were found to respond only to 200 nm sized nanorods. Endothelial cells, which line blood vessels and mediate vascularization of implant sites, were found to respond to feature sizes greater than 55 nm. Constatutive linear regression models were developed using the present empirical observations to correlate substrate nanotopography with resultant cellular morphology. This quantitative description of the changes in cellular morphology using nondimensional analysis and the Buckingham pi theorem provided an insight into engineering of cell morphology using nanopatterns on Pt-BMG structures. Fibroblasts were further analyzed for changes in focal adhesion formation and intracellular GTPases to explore molecular mechanisms underlying nanopattern-induced cytoskeletal remodeling. Consistent with changes in cell spreading, collagen production was reduced when fibroblasts were grown on nanopatterned BMGs. Finally, focused ion beam scanning electron microscopy (FIB-SEM) was employed to quantify cellular traction forces exerted by the contractile fibroblast cells with nanoscale precision.

RESULTS AND DISCUSSION

Fabrication and Characterization of Nanopatterned BMGs. Platinum-based BMG alloys (Pt-BMGs) offer significant advantages for use as a nanopatterned biomaterial due to demonstrated biocompatibility and an unprecedented combination of elasticity, strength, and ductility.\textsuperscript{9} Additionally, Pt-BMGs have demonstrated high resistance to surface oxidation during thermoplastic forming in air that is ideal for use as a biomaterials.\textsuperscript{16} In this work, arrays of nanorods were formed on Pt-based BMG substrates via thermoplastic forming of Pt\textsubscript{57.5}Cu\textsubscript{14.7}Ni\textsubscript{5.3}P\textsubscript{22.5} using alumina templates with nominal pore sizes ranging from 55 to 200 nm, termed “BMG-55”, “BMG-100”, “BMG-150”, and “BMG-200”, respectively (Figure 1). In contrast to traditional metal-forming processes that require processing at elevated temperatures to form surface patterns and manipulate microstructures, Pt-BMGs are processed at low temperatures (below 300°C) in air, similar to thermoplastics (Figure 1A).

Thermoplastic forming of BMGs in air is impacted by several parameters including maximum applied force during forming, duration of the applied force, and viscosity of the BMG (Figure 1C). The viscosity of BMGs is dependent not only on the precision of the BMG composition, but also on the crystallization kinetics dictated by the critical cooling rate during quenching as part of the BMG casting process.\textsuperscript{17} In order to produce BMG substrates with reproducible aspect ratios and substrate sizes for use in this study, the processing parameters of the TPF process were modified to account for slight batch-to-batch variation in the as-cast Pt-BMG material.

Since in excess of 100 samples were utilized for the present study, extensive quality control measures were used to ensure the fidelity of the nanopatterned substrates. Specifically, SEM characterization of the nanopatterned substrates was completed for each batch of samples to verify consistent nanotopography. In addition, EDX was employed to confirm the integrity of the nanopattern surface, the absence of residuals from the KOH wet etch process, and other contaminants.

Growing evidence suggests that surface topography presents biomechanical cues that are important in regulating cell behavior, which can be adjusted to manipulate cell functions such as protein production, contractility, morphology, migration, focal adhesion formation, and stem cell differentiation.\textsuperscript{1–5,18} Nanopatterned Pt-BMGs provide a unique platform to investigate these phenomena in vitro because modified surfaces can be fabricated with unprecedented precision. Moreover, substrate stiffness as perceived by cells can be modified without compromising the mechanical properties of the bulk material.\textsuperscript{19} Therefore altering the surface topography by engineering nanoscale structures is a means to modify the effective surface
stiffness to be lower than that of conventional bulk materials. Thus, by modulating the effective surface stiffness, the negative consequences of mechanical compliance mismatch between tissue and implants can be ameliorated.

**Cell Responses to Nanopatterned BMGs.** Influence of nanotopography on cellular morphology was assessed by monitoring cytoskeletal remodeling on nanopatterned Pt-BMG substrates. Cell adhesion and survival were supported by all nanopatterned Pt-BMGs (Supporting Information, Figure S1). To monitor changes in cell morphology induced by nanopatterns, cells were seeded onto patterned or flat control BMGs for 24 h. Cells were subsequently fixed, stained, and imaged using fluorescence microscopy. Changes in the morphology of fibroblasts, bone marrow-derived macrophages, and human umbilical vein endothelial cells (HUVECs), all of which are relevant to FBR and tissue regeneration processes, were evaluated by examining actin cytoskeletal remodeling. Distinct changes were observed for all cell types, with the resulting cellular morphologies dependent on nanorod dimensions (Figure 2 and 3). Fibroblasts became increasingly smaller, more circular, and less elongated on the BMGs with increasing nanopattern feature size. This behavior suggests that fibroblast mechanosense and respond to surface topography for all studied nanopatterns. Interestingly, macrophage morphology on the flat BMG control was similar to that on BMG-55, BMG-100, and BMG-150. In contrast, macrophage morphology differed appreciably on BMG-200 nanopattern, indicating that within the range utilized in these studies the smallest feature size to induce morphological changes (e.g., approximately 50% change) in macrophages is 200 nm. HUVECs on flat BMG and BMG-55 were similar in area, but
had lower area, perimeter, elongation factor, and higher circularity on BMG-100, BMG-150, and BMG-200 substrates. These observations suggest that nanostructures greater than 55 nm are required to induce morphological changes in HUVECs. Using the criterion of actin cytoskeleton remodeling in fibroblasts, macrophages, and HUVECs, these studies show that nanopattern feature size detection is cell type-dependent.

Cytoskeletal remodeling induced by nanotopography has been described previously. Nanorods have been shown to induce a decrease in fibroblast cell spreading, which is consistent with the findings of the present study. Conflating reports exist on the influence of nanotopography on endothelial cell spreading, but high aspect nanorods (as opposed to low aspect ratio nanorods) have been shown to...
decrease cell spreading, similar to our findings.\textsuperscript{26,27} Although randomly oriented nanorods have been shown to decrease macrophage cell spreading, there is some evidence to suggest that nanotopography can induce cell spreading in macrophages as well.\textsuperscript{28,29} While changes in cellular morphology induced by nanotopography have been described before, the current study provides a systematic examination of well-characterized high aspect ratio nanorods with feature sizes in the range of 55 to 200 nm to identify cell type-specific responses.

Biomaterial acceptance/rejection is mediated primarily by macrophages, and the subsequent fibrotic response by a variety of cell types including fibroblasts and endothelial cells. To design surface nanopatterns that can limit biomaterial rejection and increase implant longevity, it is important to differentially engineer multiple cell types \textit{in vivo}. The present results indicate that some cells such as fibroblasts can detect nanopatterns as small as 55 nm in size, while macrophages do not respond to nanopatterns smaller than 150 nm. Characterization of these cell type-specific responses to feature sizes will be critical in order to hone in on the range of effective nanopatterns relevant for specific \textit{in vivo} applications. The ability to induce specific responses will also be important in the design of nanopatterns that engineer one cell type and not affect other cell types in the microenvironment. Such work will also help identify relevant length scales for further investigation of the role of nanotopographical cues inherent in healthy and diseased tissue, and associated extracellular matrix. Along with examining the effect of nanotopography on cell function, consolidative efforts toward characterizing cell type-specific detection limits will be key to understanding and exploiting cell—nanotopography interactions for biomedical applications.

Quantitative Correlations between Cell Morphology and BMG Nanotopography. Using nondimensional analysis based on the Buckingham pi theorem, changes in cell morphology and substrate nanotopography were quantitatively correlated (Supporting Information II). Multiple linear regression was employed to describe the correlation among three nondimensional variables: percent change in cell circularity induced by nanopatterns ($S$), nanorod dimension parameter ($N$), and nanorod surface area parameter ($SA_N$). Fibroblast circularity correlated to BMG nanotopography as follows:

$$S = -9485.79 (N) + 2.96 (SA_N) + 0.25$$  \hspace{1cm} (1)

Analysis can be extended to correlate substrate nanotopography to cellular protein expression and genetic profiles, ultimately leading to informed design of nanopatterned biomaterials to induce anti-inflammatory and regenerative cellular phenotypes.

Biomechanical Cues Underlying Cell—Nanopattern Interactions Govern Cell Activity. The complex interactions between cells and nanorods required a multifaceted approach to investigate the potential mechanisms dictating the cytoskeletal remodeling observed by fluorescence imaging. Biochemical assays were performed to investigate the molecular basis for changes in cell morphology. Additionally, novel electron microscopy techniques were employed to image cell—nanorod interactions and quantify cellular traction forces with nanoscale precision. Fibroblasts were selected for these additional analyses since they exhibited changes in morphology when exposed to the entire range of nanopatterned BMG substrates. Additionally, fibroblasts are critical to the foreign body response as primary contributors of the fibrotic encapsulation that often renders implantable materials nonfunctional.

In order to investigate the molecular mechanisms underlying fibroblast—nanopattern interactions, nanopattern-induced changes in adsorption of the serum proteins, formation of focal adhesions, expression of downstream effector Rho-A GTPase, and collagen production were analyzed. Changes in BMG nanopattern dimensions consequentially modulated the extent and distribution of fibronectin absorption from serum-containing culture media (Supporting Information, Figure S2). Modifications of focal adhesion formation in fibroblasts in response to nanopatterns were analyzed by immunofluorescent detection of paxillin. Focal adhesion density increased with increasing nanopattern feature size, corresponding to the reduced size of fibroblasts on nanopatterned BMGs (Figure 4AC). In addition, intracellular levels of active Rho-A, a small GTPase that regulates actin remodeling, were significantly lower in fibroblasts grown on BMG-55s and BMG-200s in comparison to flat BMG control (Figure 4D).

Furthermore, fibroblasts grown on nanopatterned substrates were observed to produce significantly less collagen-I than fibroblasts grown on flat BMGs (Figure 4B,E). Nanopattern-induced reduction in spreading of fibroblasts corresponded to a change in the fibrotic phenotype of these cells. Collagen-I production by fibroblasts leads to fibrosis in diseased tissue as well as encapsulation of biomaterials \textit{in vivo}, leading to implant failure. These results indicate that nanopatterning of BMGs can influence cell function by significantly inhibiting matrix production by fibroblasts.

Taken together, these results suggest that biomechanical cues mediate cellular sensing of substrate nanotopography, which therefore influences cell function. Varying the nanopattern dimensions alters the adsorption patterns of serum protein and effective substrate stiffness, leading to changes in cellular focal adhesion formation. Changes in focal adhesion density, which correspond to effective nanorod stiffness, lead to changes in intracellular cytoskeletal remodeling proteins. Specifically, increasing focal adhesion density corresponds with decreasing active Rho-A GTPase levels, resulting in reduced cell spreading and
decreased collagen production. By modifying cell function through altering the fibrotic response, nanopatterned BMGs may provide a viable approach to control biomaterial rejection and increase implant lifetime.

**Cellular Traction Force Measurement.** To gain additional insight into the mechanical mechanisms underlying the cell–nanorod interactions, scanning electron microscopy (SEM) was employed. Analysis of cells with this method corroborated the trends in cell spreading observed using fluorescence microscopy. In addition, extensive bending of the nanorods at the cell perimeter was revealed, as reported previously with other patterned substrates (Supporting Information, Figure S3). To examine nanorod bending not visible underneath the cell surface, focused ion beam (FIB) milling was employed to cross-section fibroblasts on nanorods in situ (Figure 5A–F). FIB cross-sections revealed no penetration by fibroblast cells into the internanorod space, suggesting that the cells

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**Figure 4.** Molecular mechanisms underlying fibroblast–nanopattern interactions. (A) Representative images of fluorescently labeled paxillin (green) and cell nuclei using DAPI (blue) in 3T3 fibroblast cells grown for 24 h on BMGs. (B) Representative images of 3T3 fibroblasts fluorescently labeled for collagen I (green) and DAPI (blue). (C) Cells were analyzed for each condition to quantify the focal adhesion fraction and (D) Rho-A activation. (E) Quantification of collagen production in fibroblasts grown on BMGs using the integrated fluorescence intensity (*p < 0.05, p < 0.005, t-test, comparison to value of corresponding parameter for flat BMG control). Error bars represent the standard error of mean. Scale bar, 20 μm (A,B).
were sensing and responding to the nanotopography rather than the bulk BMG substrate.

Cell spreading and migration occur in a dynamic environment; therefore, elastic bending of the nanorods is important to ensure that cells perceive consistent nanotopography throughout the lifetime of an implant or device. On the basis of analysis of fibroblasts on BMGs, the resulting maximum stress of approximately 400 MPa exerted on the nanorods was less than the 1400 MPa yield strength of the Pt-BMG alloy. Elastic bending was corroborated further by recovery of erect nanorods following cell removal using surface ion milling (Figure 5C).

Nanorod deflection was observed to increase radially extending outward from the cell, and analysis of the nanorod deflections allowed cellular traction forces to be quantified with nanoscale precision. Local force exerted on the nanorods was determined using the measured individual rod deflections and nominal bending stiffness (Supporting Information I). On the basis of local force calculations, individual traction forces were compiled to form traction force maps with nanoscale resolution for fibroblasts on both BMG-55 and BMG-200 substrates (Figure 5E,F). The maximum calculated deflection of the nanorods was observed to be approximately equivalent for both the BMG-55 and...
BMG-200 substrates. Despite differences in the height of the nanorods between the respective substrates, the stiffness of the individual nanorods composing the substrates is approximately equivalent since the aspect ratios of the BMG-200 and BMG-55 substrates were designed to be constant (Figure 1C, Supporting Information I). Therefore, since fibroblasts perceive the same nanorod stiffness, the forces exerted by the cell on the nanorods at the cell perimeter produce a comparable magnitude of deflection for both the BMG-200 and BMG-55 nanopatterns.

While several studies of cell traction forces on microscale structures have proven useful to quantify intracellular forces, such methodologies cannot be extended to the nanoscale because of challenges with resolution. Therefore, the FIB traction force mapping technique provides unprecedented insight into cell–nanopattern interactions in terms of both topography and scale. Conceivably, this methodology may be extended to build more complex traction force maps by integrating data from multiple FIB cross-sections of the same cell. A wide range of values for cellular traction forces, ranging from 1–100 nN for polymeric substrates to 4.7 μN for silicon, have been reported in the literature. Traction force is a function of nanorod stiffness that scales linearly with the elastic modulus of the substrate material (Supporting Information I). Therefore the variation in reported traction force correlates with the elastic modulus of the substrate material, which can range in magnitude from 2 MPa for polymers such as PDMS to 151 GPa for silicon. Since Pt57.5Cu14.7Ni5.3P22.5 has an elastic modulus of 94.8 GPa, the maximum observed cellular traction force value of 1.2 μN (Figure 5E,F) is consistent with the previously reported values. These trends illustrate the dynamic reciprocity between traction forces imparted by the cell and the inherent material properties of the substrate and associated nanopatternography.

CONCLUSION

In summary, this study highlights how nanopatterned BMGs comprise a versatile class of materials for load bearing or structural implants that incorporate tunable surface features to drive cell-specific behaviors without the use of exogenous growth factors. Specifically, the detection of nanopatterns varied markedly according to cell type, ranging from as high as 200 to 55 nm or lower. Additionally, the characterization of the sensitivity of cells to nanopatterns will be crucial for designing biomaterials that could selectively influence multiple cell types in vivo. In addition, novel FIB microscopy techniques enabled traction force measurements and the examination of cell–nanorod interactions with nanoscale precision. Coupled with nondimensional statistical analysis, these results provide new insights into stiffness-induced cell behaviors, the limits of biomechanical detection in a variety of cells, and a foundation for rational design of nanopatterned materials to attenuate biomaterial rejection. This approach can be extended to analyze the response of other relevant cell types associated with tissue regeneration and FBR, including mesenchymal stem cells. Thus, nanopatterned BMGs can be used to design cell-instructive constructs that retain bulk material integrity for use in biomedical applications.
RESULTS AND NOTES

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