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Design and Fabrication of Complex Micro–Nano Structures for Osteogenesis Interfacing in 3D

Xuanrui Zhou¹ | Yahui Pan^{1,2,3} | Thomas Grap⁴ | Sven Ingebrandt¹  | Ziyu Gao¹ 

¹Institute of Materials in Electrical Engineering 1, RWTH Aachen University, Aachen, Germany | ²Biomaterials and Tissue Engineering Group, Department of Oral Biology, University of Leeds, WTBB, St. James's University Hospital, Leeds, UK | ³Nanjing Stomatological Hospital, Affiliated Hospital of Medical School, Institute of Stomatology, Nanjing University, Nanjing, China | ⁴Helmholtz Nano Facility (HNF), Forschungszentrum Jülich, Jülich, Germany

Correspondence: Ziyu Gao (ziyu.gao@iwe1.rwth-aachen.de)

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ABSTRACT

Engineered surface topographies at the micro- and nanoscale have been shown to effectively regulate physical behavior and biochemical signaling of mechanically sensitive cells, e.g., bone-related cells, especially in 3D. However, scalable and cost-effective methods to fabricate biologically instructive 3D silicon structures remain limited. The deep reactive ion etching (DRIE) process, although widely used in microelectromechanical systems, has not been fully explored for constructing biomimetic substrates that support in vitro bone-related cell cultures. By extending the application of DRIE beyond conventional micromechanical uses, this study introduces a scalable and tunable strategy for constructing functional micro–nano structures suitable for tissue engineering and biointerface research. Several designs and fabrication protocols for a variety of 3D topographic structures, including obelisks, scalloped pillars, and wine glass shapes were developed. Wafer-scale fabrication was achieved with the DRIE technique, particularly with high controllability at micro- and nanoscale for structural height, dimension, and sharpness. By adapting the classic DRIE process to fabricate silicon microstructures with tailored topographies, the resulting approach offers new possibilities for designing and fabricating 3D in vitro cell culture scaffolds, which can be applied for in vitro biomechanical studies, such as cell-chip coupling, cellular guidance, and differentiation.

1 | Introduction

Native bone tissue exhibits a highly hierarchical 3D architecture in which microscale curvature, undercut features, and anisotropic fibrillar structures collectively regulate cellular adhesion, mechanotransduction, and matrix deposition [1, 2]. Cells in vivo continuously interpret 3D structural cues through integrin-mediated adhesion complexes that couple extracellular geometry to intracellular signaling pathways [3]. In osteoprogenitor cells, changes in spreading area, curvature sensing, and anisotropic confinement directly regulate actomyosin contractility and downstream mechanotransductive pathways such as YAP/TAZ

and focal adhesion kinase signaling [4, 5]. Structured interfaces that introduce controlled curvature transitions, undercut side-walls, and vertical dimensionality can therefore amplify cell–material coupling beyond what is achievable with planar or simple pillar arrays [6].

In particular, concave–convex geometries and anisotropic ribbon-like elements are known to modulate cytoskeletal tension, focal adhesion distribution, and spatial confinement, thereby influencing osteogenic differentiation [7, 8]. Concave features can enhance local cell anchoring and mechanical interlocking [9], while flared or tapered geometries generate gradients

Xuanrui Zhou and Yahui Pan contributed equally to this study.

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in curvature and stress distribution that guide cytoskeletal organization. Similarly, ribbon-like structures provide directional guidance cues, promoting cell elongation and alignment, morphological signatures that are closely associated with osteogenic commitment [10]. These structural characteristics suggest that deliberately engineered wine glass-shaped and ribbon-inspired microarchitectures may serve as powerful physical regulators of osteogenesis. Beyond early adhesion events, defined 3D structural features may also influence extracellular matrix organization and mechanical coupling at the interface, thereby sustaining osteogenic functionality over time [11].

Despite increasing recognition of geometry-driven osteogenesis, most existing platforms rely on simplified cylindrical pillars [12, 13], microgrooves [14], or nanopits [15] fabricated through multistep modification processes. These approaches often lack true 3D complexity, structural tunability, and scalable reproducibility. Importantly, generating controlled undercut profiles, curvature gradients, and high-aspect-ratio ribbon geometries in a wafer-scale manner remains technically challenging. Therefore, there is a pressing need for fabrication strategies capable of producing complex, hierarchically tunable 3D micro-nano structures specifically designed to regulate osteogenic interfacing.

Silicon-based microfabrication offers a robust and scalable route for precisely constructing defined topographies while enabling integration with multifunctional platforms for multimodal readout and localized stimulation [16–18]. Techniques such as electroplating, atomic layer deposition [19], and two-photon polymerization [20] have been employed to construct biointerfaces; however, each technique presents limitations in structural precision, throughput, or scalability [21]. In contrast, deep reactive ion etching (DRIE), also known as the Bosch etching process, provides high-aspect-ratio patterning such as microchannels [22, 23], microneedles, and electrode arrays [24, 25] with excellent reproducibility while being compatible with complementary metal-oxide-semiconductor processes [26, 27]. By alternating etching and passivation cycles, the Bosch process enables controllable vertical profiles and complex geometries, including tapered and undercut structures [27–29], at micrometer precision across wafer-scale substrates [30, 31]. These characteristics make it particularly suitable for engineering structurally complex and scalable 3D biointerfaces [32, 33].

In this work, we establish a design–fabrication framework for constructing complex 3D micro–nano silicon structures tailored for osteogenic interfacing. By engineering wine glass-shaped and ribbon-inspired geometries that introduce controlled curvature, anisotropy, and undercut profiles, we created structurally defined microenvironments capable of modulating cell spreading, cytoskeletal organization, and osteogenic behavior. Wafer-scale processing ensures scalability and reproducibility, while micro–nano structural tuning enables systematic investigation of geometry-dependent cellular responses.

Finally, *in vitro* cellular studies confirm that these engineered 3D architectures elicit distinct, structure-dependent osteogenic responses, demonstrating that rational geometric design combined with controllable fabrication provides a powerful strategy for constructing bioinstructive 3D interfaces. Specifically, the

wine glass-shaped structures, characterized by tapered necks and widened heads, demonstrated enhanced mechanical interlocking and localized force concentration, while ribbon-like elements provided anisotropic mechanical environments that stabilize elongated cytoskeletal architectures favorable for osteogenic differentiation.

This work expands the scope of Bosch-based microfabrication from conventional mechanical applications toward biologically functional micro–nano engineering, offering a scalable pathway for next-generation osteogenic platforms.

2 | Materials and Methods

2.1 | Structure and Layout Design

Various silicon scaffold layouts were designed with three different diameters: 3, 4, and 5 μm . For each diameter, three different densities were implemented, determined by center-to-center spacing between neighboring scaffolds, referred to as the pitch (P8, P12, and P20). The scaffolds were arranged into arrays, each labeled according to its diameter and pitch. For example, an array with a 4 μm diameter and a 20 μm pitch is denoted as D4P20. Each array was duplicated on a single sample for consistency.

2.2 | Mask Fabrication with Photolithography

The fabrication process was carried out on 4-inch silicon (Si) wafers (Micro Chemicals, Germany). To improve the adhesion between the photoresist and the wafer surface, the wafers were pretreated with hexamethyldisilazane (HMDS) (Micro Chemicals, Germany). Specifically, a carrier wafer was first placed on a hot plate, and the sample wafer was then positioned on top of the carrier wafer. The hot plate was heated to 135 $^{\circ}\text{C}$, and the chamber lid was closed to initiate vacuum conditions, enabling the HMDS coating process to proceed uniformly.

Following HMDS treatment, a 1.5 μm -thick layer of AZ5214E photoresist (Micro Chemicals GmbH, Germany) was spin-coated onto the wafer surface at a speed of 5000 rpm. The coated wafer was then soft-baked at 105 $^{\circ}\text{C}$ for 1 min to remove residual solvents.

Photolithography was performed using a direct Laser Writer 405D (Microtech, Italy), exposing the photoresist with a wavelength of 405 nm and an exposure dose of 314 mJ/cm². The exposed wafers were developed in AZ726MIF developer (Micro Chemicals GmbH, Germany) for 1 min, followed by soaking in deionized water under microwave agitation for 5 min to ensure complete removal of development residues.

2.3 | Si Structure Fabrication with Reactive Ion Etching

1 $\times$ 1 cm silicon chips were used for the initial process development. To prevent local overheating from plasma bombardment, these chips were mounted on a carrier wafer with a drop of

Fomblin oil applied between them to ensure efficient heat transfer to the cooled chuck. Once the etching parameters were optimized, the final structures were fabricated directly on 4-inch silicon wafers. These full-size wafers were loaded directly into the chamber, without carrier wafers and Fomblin. The patterned wafers were processed using DRIE in a PlasmaPro 100 Cobra system (Oxford Instruments, UK). The Bosch process began with the introduction of SF₆ and C₄F₈ gases into the chamber, followed by plasma ignition using a two-step strike sequence. During these steps, the gases are dissociated into reactive species such as SF_x⁺, F* radicals, and C₂F₄* fragments. This was followed by multiple etch-passivation cycles, in which alternating steps of etching and deposition shape the silicon structure. After completing the specified number of cycles, the system underwent a 30-s pumping step to evacuate residual gases and byproducts.

Before processing the actual samples, a dummy wafer was etched to condition the chamber. Between each processed sample, an intermediate chamber cleaning was performed using a gas mixture of sulfur hexafluoride (SF₆) and oxygen (O₂) to remove potential polymer buildup. After completing the etching process, an additional O₂ plasma clean step was conducted at 45 °C for 15 min to ensure the chamber was free of residual contamination.

The etching selectivity (*r*) was calculated using the following equation

$$r = \frac{H}{h_1 - h_2} \quad (1)$$

where *H* is the height of the structures, *h*₁ is the thickness of the photoresist before etching, and *h*₂ is the thickness of the photoresist after etching.

2.4 | Morphology Characterization

The fabricated samples were diced into square chips (2 × 2 cm) and characterized using scanning electron microscopy (SEM) (Zeiss Sigma) for morphological and topographical characterization. Images were taken with 5 kV accelerate voltage without additional metal coating. To assess the sidewall structures, the samples were tilted by 5° during SEM imaging, revealing the alignment of the arrays according to the designed pitch ratios.

2.5 | Evaluation of Cell Viability and Osteogenesis on Wine Glass-Shaped Silicon Substrates

The wine glass-shaped chip was fabricated as previously described. For biological evaluation, we selected four designs (D3P8, D4P8, D5P8, and D4P8T) to assess cell viability after 24 h of culture and used D3P8 and D5P8 to preliminarily investigate osteogenic potential. Blank silicon wafers without microstructures were used as control substrates.

The human osteosarcoma cell line G292 (ATCC CRL-1423) was used for all experiments. Cells were cultured in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco), and maintained

at 37 °C in a humidified incubator with 5% CO₂. Cells were seeded at an appropriate density onto the surface of the chips and cultured for 24 h prior to viability staining.

Cell viability was assessed using LIVE/DEAD Viability/Cytotoxicity kit (Thermo Fisher Scientific, Cat. No. R37601), following the manufacturer's instructions. After staining, samples were imaged using a fluorescence microscope (Carl Zeiss, Axio Observer 5). Live cells appeared green, while dead cells appeared red. Cell coverage, aspect ratio, circularity, roundness, and orientation were used as indicators for cell density and morphology evaluation. Fluorescence images were acquired from five randomly selected regions for each sample. The same thresholding was applied to all groups.

To assess the osteogenic potential of the microstructured substrates, G292 cells were subjected to osteogenic induction for 7 days. Following induction, alkaline phosphatase (ALP) staining was performed to visualize early osteogenic differentiation. Cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature, washed with distilled water, and incubated with ALP staining solution (Naphthol AS-MX phosphate and Fast Violet B) at 37 °C in the dark for 60 min. After washing, cells were imaged, and red staining indicated ALP activity. To provide a semiquantitative comparison of osteogenic staining among different micro-nano structures, ALP-stained images were analyzed using ImageJ under identical thresholding conditions. Two parameters were quantified: ALP-positive staining area and background-corrected relative staining intensity. The ALP-positive area was identified using the same threshold range for all images and expressed as a percentage of the total analyzed area. For intensity analysis, the mean gray value of the whole analyzed image was measured and normalised to the corresponding background gray value.

In addition, SEM was conducted after osteogenic induction to observe cell morphology and attachment on the microstructured surfaces. Samples were fixed with 4% PFA, followed by dehydration at 4 °C using a graded ethanol series: 30% (10 min), 50% (10 min), 70% (10 min × 2), 95% (10 min × 3), and 100% (10 min). After complete ethanol removal, samples were air-dried. Finally, samples were sputter-coated with gold prior to SEM imaging.

2.6 | Data Analysis and Statistics

All qualitative observation of the fabricated structures were reported after at least three independent repetitions on the fabrication process. The quantitative data were calculated and plotted after ImageJ analysis (ImageJ 1.53t, USA). Cell orientation image was generated using MATLAB (R2024a, USA).

All the biological data points were presented in mean ± SD with sample numbers varying from 3 to 6. Statistical analyses were performed using GraphPad Prism (version 10.5, USA). For normally distributed data, group differences were evaluated using one-way analysis of variance followed by Tukey's post hoc test. For data that did not meet normality assumptions, the Kruskal-Wallis test followed by Dunn's multiple comparisons test was applied.

3 | Results and Discussion

3.1 | Obelisk-Shaped Si Structures

The fabrication of the obelisk-shaped structures was based on Recipe 1 (in Table S1), with a total of eight process variants designed to systematically optimize the structural profile. Variants 1.1–1.4 were first used to investigate the effect of varying high-frequency power (from 20 to 80 W) during the etch step (Tables S1–S4). Theoretically, increasing HF power enhances vertical ion bombardment, which increases the etch depth but concurrently induces stronger secondary scattering that exacerbates lateral erosion on the sidewalls. To mitigate this excessive lateral erosion while still achieving taller structures, we strategically fixed the HF power at a conservative value of 20 W for Variants 1.5–1.8. Under this minimized scattering condition, we explored the effect of extending the etch duration (from 5 to 20 s) to compensate for the lower etch rate and proportionally scale the structure height (Tables S5–S8). The resulting structures and their morphological evolutions are presented in the SEM images (Figure 1a–j).

Variant 1.1, using the initial recipe with 40 cycles, resulted in an etch depth of only ~ 200 nm. The sidewall angle could not be clearly identified, and the photoresist layer remained largely unaffected. As HF power increased from 20 to 80 W, etch depth increased correspondingly, reaching ≈ 800 nm at 80 W. However, instead of the near-vertical sidewalls typically produced by conventional Bosch processes, the resulting structures exhibited a tapered profile, characterized by narrower tops and wider bases. At 80 W, the measured sidewall angle was $\approx 73^\circ$ (Table 1).

In Variants 1.5–1.8, the HF power was fixed at 20 W, while the etch time was increased from 5 to 20 s. Etch depth increased accordingly, from $\approx 1.5 \mu\text{m}$ at 5 s to $6.6 \mu\text{m}$ at 20 s. As shown in Figure 1h, the resulting structure displays a pronounced obelisk shape.

Across all eight variants, the measured sidewall angles ranged from 72° to 77° . Considering possible measurement uncertainties, this indicates that the sidewall angle remained relatively consistent and was not significantly influenced by either HF power or etch duration. These structures did not exhibit the characteristic sidewall scallops typically observed in conventional Bosch etching. Instead, the etched profiles presented smooth, sloped surfaces.

The HF power in the strike stage was too low; as a result, the plasma was not ignited, leading to insufficient plasma generation, which caused the etching rate to be very low. Additionally, due to the low HF power, an insufficient amount of CF_2^* was formed, preventing the upper parts of the sidewalls from obtaining an adequate protective layer. Theoretically, the HF power primarily affected vertical ion bombardment, mainly targeting the trench bottom. However, when HF power was excessively high, the energy and density of ions in the plasma became too intense, potentially causing secondary scattering effects that deflect some ions toward the sidewalls, thereby etching them.

Moreover, high-energy ions in the plasma can be reflected and scattered as they propagate within the trench, causing partial

ion bombardment of the sidewalls. The resulting increase in sidewall etching ultimately produces an “inverted taper” or “obelisk” shape. Furthermore, during the deposition step in the Bosch process, the protective layer was not always evenly distributed across the top and bottom regions. As the trench deepened, the coverage thickness of the protective gas (e.g., C_4F_8) on the trench bottom and sidewalls may have differed. The protective layer tended to deposit more effectively on flat trench bottoms, while the sidewalls, especially the upper portions, often had thinner protective layers. Consequently, when the HF power increased, the thinner protective layer on the upper sidewalls became more susceptible to over-etching, further contributing to the formation of the “obelisk” shape.

3.2 | Pillar-Shaped Si Structures

To overcome the limited etching depth and the absence of scallop patterns observed in Recipe 1, we developed Recipe 2. The previous obelisk profiles were primarily caused by insufficient HF power during the initial “strike” stage, which led to unstable plasma ignition and inadequate gas dissociation. Therefore, we increased the initial strike HF power to 75 W (Table S9). This high-power adjustment ensures rapid and stable plasma ignition. By generating a high density of reactive radicals right from the start, it effectively supports the regular alternating etch-and-deposition cycles of the Bosch process, successfully achieving deeper anisotropic etching with well-defined scalloped profiles.

As shown in Figure 2a, after 10 etching cycles, the upper portion of the silicon pillars exhibited distinct scallop patterns, while the lower portion presented irregular features, indicating localized over-etching near the base. Following 20 cycles, the photoresist at the top of the structures showed pronounced degradation, and the undercutting at the bottom became more severe (Figure 2b).

To mitigate these issues, a drop of Fomblin oil was applied between the carrier wafer and the diced chip sample. Fomblin can ensure efficient heat dissipation and significantly improve thermal uniformity during the process, thereby thermodynamically stabilizing the deposition of CF_2 radicals during the passivation cycles [34]. In the absence of Fomblin, the rapid consumption of the mask at elevated temperatures resulted in a critically low etch selectivity between the silicon substrate and the photoresist, ranging from 3.42 to 4.17 (Table 2). Conversely, the excellent heat transfer efficiency provided by Fomblin preserved both the mask and the sidewall passivation layer, substantially increasing the etch selectivity to 48.1. Furthermore, without Fomblin, localized heat accumulation over increasing etching cycles severely degraded the polymer passivation layer. As observed from the progression between Figure 2a (10 cycles) and Figure 2b (20 cycles), this resulted in severe undercutting with a sidewall angle of 82.9° . Specifically, by the final few cycles of the 20-cycle process (Figure 2b), the scallop patterns at the bottom of the pillars became practically unrecognizable. After applying Fomblin, undercutting was effectively suppressed, and the sidewall angle improved from 82.9° to 90.0° . Consequently, even after 50 etch–deposition cycles, the scallop patterns remained clearly defined along the entire deep sidewall (Figure 2c).

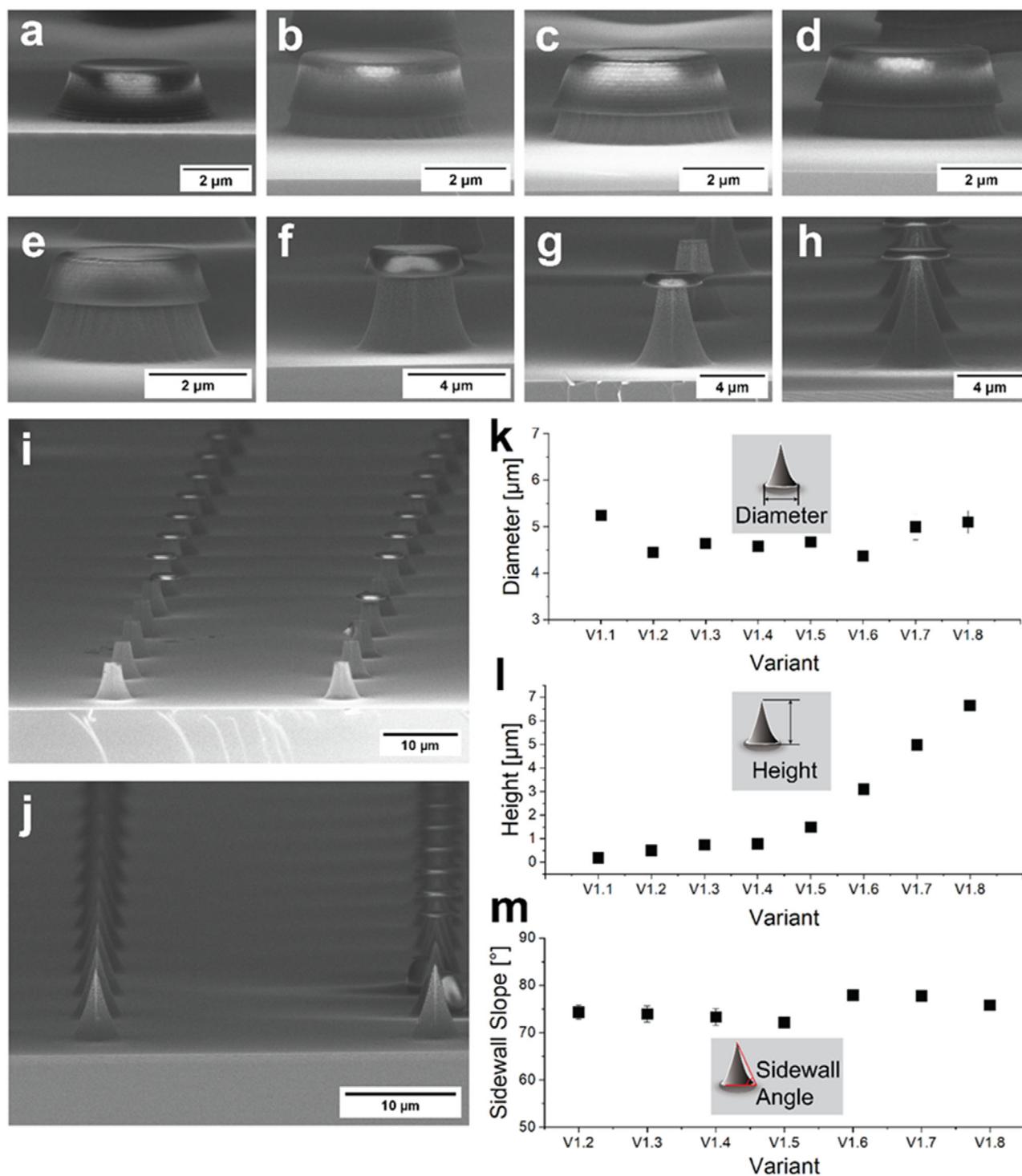


FIGURE 1 | SEM images of the “Obelisk” structures fabricated using the eight different process variants based on the recipe 1. (a–d) Structures obtained with HF powers of (a) 20 W, (b) 40 W, (c) 60 W, and (d) 80 W (40 cycles), respectively. (e–h) Structures obtained with etch step durations of (e) 5 s, (f) 10 s, (g) 15 s, and (h) 20 s, with HF power fixed at 40 W (40 cycles), respectively. (i, j) Top-view images of arrays fabricated with (i) Variant 1.7 and (j) Variant 1.8. (k–m) Quantitative plots of (k) diameters, (l) heights, and (m) sidewall angles corresponding to each variant.

3.3 | Wine Glass-Shaped Si Structures

Recipe 3 was developed based on Recipe 2, with the aim of creating structures optimized for biological interfacing. As shown in Figure 3, the resulting geometry resembles a wine glass, with a wider upper part (referred to as the bowl) that acts as a cap

preventing cell escape, and a narrower lower part (stem). In addition, the scalloped sidewalls increase surface area, potentially enhancing synaptic adhesion.

To eliminate the influence of geometric variations on the plasma etching behavior (e.g., the microloading effect), all process

TABLE 1 | The sidewall angle of each obelisk structure variant.

	Height, μm	Diameter, μm	Sidewall angle, $^\circ$
Variant 1.1	0.2 ± 0.030	5.2 ± 0.230	—
Variant 1.2	0.5 ± 0.020	4.4 ± 0.051	74.3 ± 1.45
Variant 1.3	0.7 ± 0.018	4.6 ± 0.073	73.9 ± 1.70
Variant 1.4	0.8 ± 0.013	4.6 ± 0.072	73.3 ± 1.78
Variant 1.5	1.5 ± 0.012	4.7 ± 0.090	72.2 ± 0.73
Variant 1.6	3.1 ± 0.026	4.4 ± 0.106	77.9 ± 0.66
Variant 1.7	5.0 ± 0.070	5.0 ± 0.279	77.7 ± 1.10
Variant 1.8	6.6 ± 0.097	5.1 ± 0.248	75.8 ± 0.65
The etching ratio between substrate and photoresist: 6.31			

optimization variants (Variants 3.1–3.5) presented in Figure 3 and Table 3 were uniformly fabricated using the D4P8 array layout.

As outlined in Table S10, Recipe 3 uniquely incorporated two separate etch–deposition loops. To achieve the distinctive wine glass geometry with a wider top (bowl) and a narrower bottom (stem), we strategically manipulated the etch-to-deposition time ratio while maintaining other gas parameters constant. Specifically, the first loop utilized a time ratio of ≈ 1.3 to define the wider bowl, whereas the second loop drastically increased this ratio to 5.0 to facilitate a deeper and narrower anisotropic etch for the stem.

The structure fabricated using this initial recipe (referred to as Variant 3.0, Figure 3a) successfully exhibited the intended wine glass profile. However, it suffered from severe localized over-etching, evidenced by irregular holes on the sidewalls. Because the continuous nature of the dual-loop process obscured whether this passivation failure originated during the first or second loop, we systematically designed five subsequent variants (Variants 3.1–3.5) to isolate the parameter effects and optimize the profile. To investigate the first loop, Variants 3.1 and 3.2 substantially increased the deposition inductively coupled plasma (ICP) power

TABLE 2 | The data of the pillar structures of each variant.

	Height, μm	Diameter, μm	Etching ratio (substrate/photoresist)
Variant 2.1	3.6 ± 0.051	4.6 ± 0.089	4.17
Variant 2.2	6.0 ± 0.056	4.3 ± 0.248	3.42
Variant 2.3	15.6 ± 0.090	4.5 ± 0.057	48.1

to 1500 W to enhance radical formation, coupled with minor etching adjustments (Tables S11 and S12). While this successfully eliminated the sidewall holes, the excessively thick polymer passivation severely restricted the longitudinal etch rate, resulting in shorter bowl regions (Figure 3b,c). Consequently, for Variants 3.3–3.5, the first loop parameters were reverted and locked as control variables. Instead, a targeted parameter sweep was performed on the deposition duration of the second loop (set to 3.0, 5.0, and 7.0 s; Tables S11–S13). All three variants demonstrated distinct scallop formation and pronounced diameter transitions between bowl and stem, as shown in Figure 3d–f.

Figure 3g shows that Variants 3.1 and 3.2 yielded a shorter bowl compared to Variants 3.3–3.5, likely due to the increased ICP power during the first deposition step. Among Variants 3.3–3.5, Variant 3.3 achieved the tallest bowl, whereas Variant 3.5 exhibited the shortest. As illustrated in Figure 3h, the tallest stem was achieved in Variant 3.3, followed by Variants 3.1 and 3.2. Variant 3.5 showed the shortest stem, consistent with its longer deposition duration. Figure 3i,j summarizes the overall dimensions: Variant 3.3 achieved the greatest total height, while Variant 3.5 exhibited the largest diameter.

By implementing two distinct etch–deposition loops, both the bowl and stem portions of the structure were formed, and their geometries could be independently controlled by adjusting the parameters of each loop. As shown in Figure 3g, Variants 3.1 and 3.2 exhibited shorter bowl regions, attributed to an increase

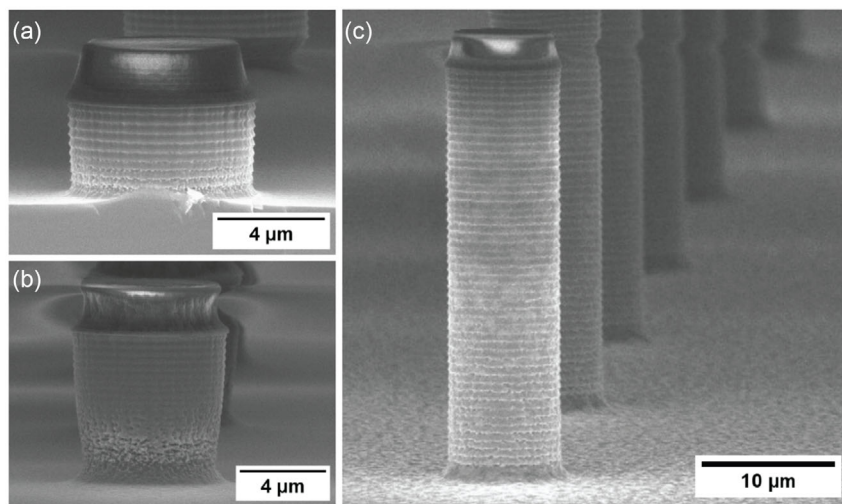


FIGURE 2 | The SEM images of “pillar” structures with three different variants based on Recipe 2. (a) The structure obtained from Recipe 2, with 10 cycles of etching and deposition. (b) The structure obtained from Recipe 2, with 20 cycles of etching and deposition. (c) The structure obtained using Recipe 2 with Fomblin coating applied between the carrier wafer and the sample, undergoing 50 cycles of etching and deposition.

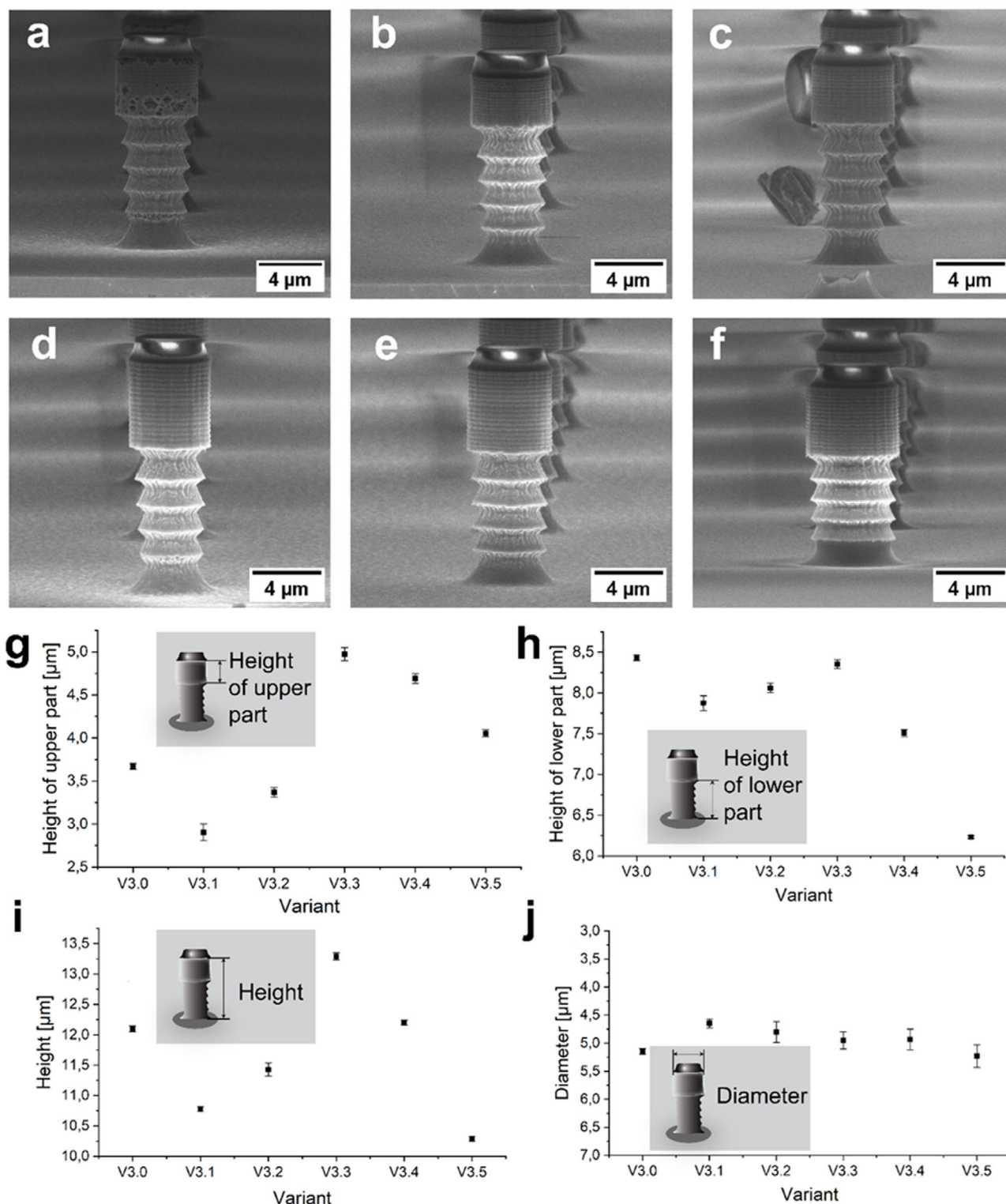


FIGURE 3 | The SEM images and quantitative analysis of “Wine glass” structures fabricated using different etching protocol variants. (a) The structure obtained with the Variants 3.1. (b) The structure obtained with the Variants 3.1. (c) The structure obtained with Variant 3.2. (d) The structure obtained with Variant 3.3. (e) The structure obtained with Variant 3.4. (f) The structure obtained with Variant 3.5. (g) All variants are described in the main text. The plot of height of upper part with each variant. (h) The plot of height of lower part with each variant. (i) The plot of the structure height of each variant. (j) The plot of diameter of each variant.

in ICP power during the deposition stage of the first loop. Higher ICP power enhanced the plasma generation, leading to increased CF_2^* radical formation, which in turn resulted in a thicker passivation layer and reduced the etching rate. Between the two,

Variant 3.1 showed a slightly taller bowl than Variant 3.2, due to its extended deposition time compared to the increased HF power applied in Variant 3.2, which enhanced ion bombardment during etching.

TABLE 3 | The data of the wine glass-shaped structures of each variant.

	Height, μm	Diameter, μm	Height of upper part, μm	Height of lower part, μm
Variant 3.1	10.8 ± 0.04	4.6 ± 0.078	2.9 ± 0.097	7.9 ± 0.092
Variant 3.2	11.4 ± 0.11	4.8 ± 0.185	3.4 ± 0.056	8.1 ± 0.056
Variant 3.3	13.3 ± 0.06	4.9 ± 0.153	5.0 ± 0.076	8.4 ± 0.054
Variant 3.4	12.2 ± 0.04	4.9 ± 0.184	4.7 ± 0.056	7.5 ± 0.043
Variant 3.5	10.3 ± 0.04	5.2 ± 0.203	4.1 ± 0.042	6.2 ± 0.018

As shown in Figure 3h,i, Variant 3.3 produced the tallest structure and the most pronounced stem section, while preserving clearly defined scallops along the sidewall. This indicates an optimal balance between etching and deposition, maximizing etch depth per cycle. In contrast, increasing the deposition duration led to reduced structure height, confirming that the total height and etch depth per cycle can be tuned by modifying the deposition-to-etching ratio.

Variant 3.5 exhibited the largest diameter; however, this was likely due to a focus or alignment error during photolithographic exposure rather than an effect of deposition time. While excessive anisotropic etching can degrade scallop definition, the scallops in Variants 3.3 and 3.4, both with relatively short deposition steps, remained well-defined. This suggests that deposition in these cases was already sufficient for sidewall protection, and the observed diameter variation is more plausibly attributed to instability or focus deviation on the Laser Writer system.

To fabricate a thinner stem and achieve a more pronounced diameter difference between the stem and bowl, the ICP power and C_4F_8 flow rate during the deposition step of the first loop can be increased to enhance the thickness of the passivation layer. Meanwhile, in the second loop, the parameters of the etching stage were adjusted by slightly reducing the HF power and increasing the SF_6 flow rate to promote more pronounced lateral etching.

3.4 | Scalable and Tunable DRIE Fabrication Protocol

The Bosch-based DRIE fabrication protocol developed in this study enabled the scalable and reproducible fabrication of complex 3D silicon microstructures at the wafer level. Three different structure types, obelisk, scalloped pillar, and wine glass-shaped, were fabricated on 4-inch wafers using a cost-effective and lithography-compatible process. The total etching time for each structure was maintained under 5 min, demonstrating the high throughput and time efficiency of the approach.

Based on the parameter optimization above, Variant 3.3 was selected as the standard recipe for wafer-scale mass production due to two main considerations. First, from a microfabrication perspective, Variant 3.3 achieved the optimal balance between etching and deposition. While effectively preventing sidewall over-etching, it minimized polymer accumulation at the trench bottom. For 4-inch wafer-scale processing, excessive deposition easily leads to uneven polymer distribution and induces severe micromasking effects. By avoiding over-passivation, Variant 3.3

ensured the chemical uniformity of the plasma across the entire wafer, thereby achieving a high fabrication yield. Second, from a biological application perspective, Variant 3.3 produced the tallest overall structure ($13.3 \mu\text{m}$), which further expands the vertical space for cell growth.

To systematically investigate the influence of scaffold size, density, and spatial arrangement on cell behavior, six array variants were designed based on the standardized Variant 3.3 recipe: D3P8, D4P8, D5P8, D4P12, D4P20, and D4P8T. Here, 'D' denotes the designed diameter of the scaffolds, and 'P' represents the spatial pitch, rigorously defined as the absolute center-to-center distance between adjacent scaffolds. By varying the diameter and the pitch, we can precisely manipulate the actual edge-to-edge gap ($\text{Gap} = \text{P} - \text{D}$), which constitutes the critical physical span that cells must overcome during cytoskeletal extension and bridging. While variants D3P8 through D4P20 were arranged in a standard 90° square lattice, D4P8T was designed with a 60° staggered arrangement, maintaining the same diameter and pitch as D4P8. Furthermore, due to the resolution limits of the photolithography equipment during wafer-scale manufacturing, the actual fabricated diameters of the scaffolds exhibited a consistent reduction of $\approx 1 \mu\text{m}$ compared to their designed values (for instance, the actual diameter of D5P8 was measured at $\sim 4 \mu\text{m}$). This systemic dimensional deviation remained highly stable across all fabricated variants, ensuring the exact relative differences in gaps and densities required for reliable downstream biological analyses.

Our optimized protocol allows precise control over key geometric parameters, including height, sidewall angle, and shape transitions, through systematic tuning of etching and passivation steps. The resulting structures were composed entirely of silicon, with high aspect ratios and submicron-level dimensional accuracy. In addition, the fabrication process remained fully compatible with downstream micro-nano processing steps, such as metallization, photolithography, and surface functionalization, thereby offering broad applicability for integration into micro-electro-mechanical systems (MEMS) or other bioelectronic devices.

3.5 | Cell Viability and Osteogenic Response on Wine Glass-Shaped Si Structures

3.5.1 | Cell Viability and Morphology

Most cells remained viable after 24 h of culture on both control and 3D scaffold arrays (Figure 4a–e), indicating that all structures were biocompatible and capable of supporting cell attachment. This observation was consistent with current findings that

various silicon-based microstructured surfaces showed no cytotoxicity and successfully supported endothelial cell adhesion [35]. On blank silicon substrates, cells exhibited a relatively round morphology, whereas cells on the 3D scaffold arrays appeared elongated and aligned along the scaffold arrangement. These qualitative observations suggest that the introduction of wine glass-shaped microstructures provides topographical cues that influence early cell spreading and organization.

The cellular adhesion and morphology exhibited pronounced geometry-dependent differences (Figure 4f–i). Although the patterned groups D3P8, D4P8, and D5P8 shared an identical center-to-center pitch of 8 μm and therefore contained the same number of scaffolds per unit area, variations in scaffold diameter resulted in substantial differences in the accessible adhesive surface. SEM imaging revealed that cells adhered predominantly to the side-walls of the re-entrant, wine glass-shaped Si scaffolds rather than the top facets, indicating that the effective adhesive interface was governed by the total available sidewall area and edge length. As both parameters increased with scaffold diameter, the scaffolds exhibited a clear adhesion hierarchy of D5P8 > D4P8 > D3P8. D5P8 supporting better cell attachment (Figure 5c3,c4), possibly

due to its longer scaffold top edge area, which had been proven to be the place where focal contacts were primarily targeting [36].

In addition to differences in adhesive surface area, the corresponding edge-to-edge gaps (3, 4, and 5 μm , respectively) further modulated cell coverage. A previous study has demonstrated that cell adhesion and spreading are highly sensitive to micrometer-scale nonadhesive gaps, with stable spanning occurring at gap sizes on the order of a few micrometers [37]. Beyond this range, increased spatial separation may reduce continuous cellular engagement with neighbouring structural features, thereby influencing cell distribution, alignment, and local substrate interaction [38]. Consistent with these reports, the reduced coverage observed on D3P8 can be attributed to gap-avoidance behavior, whereby cells minimize energetically unfavorable adhesion states across larger nonadhesive regions [39].

Morphometric analyses further confirmed the influence of the scaffold geometry on cell shape. Cells grown on wine glass-shaped micro scaffold arrays exhibited increased aspect ratios compared to the flat control (Figure 4g), consistent with the elongated phenotype induced by geometric confinement, as

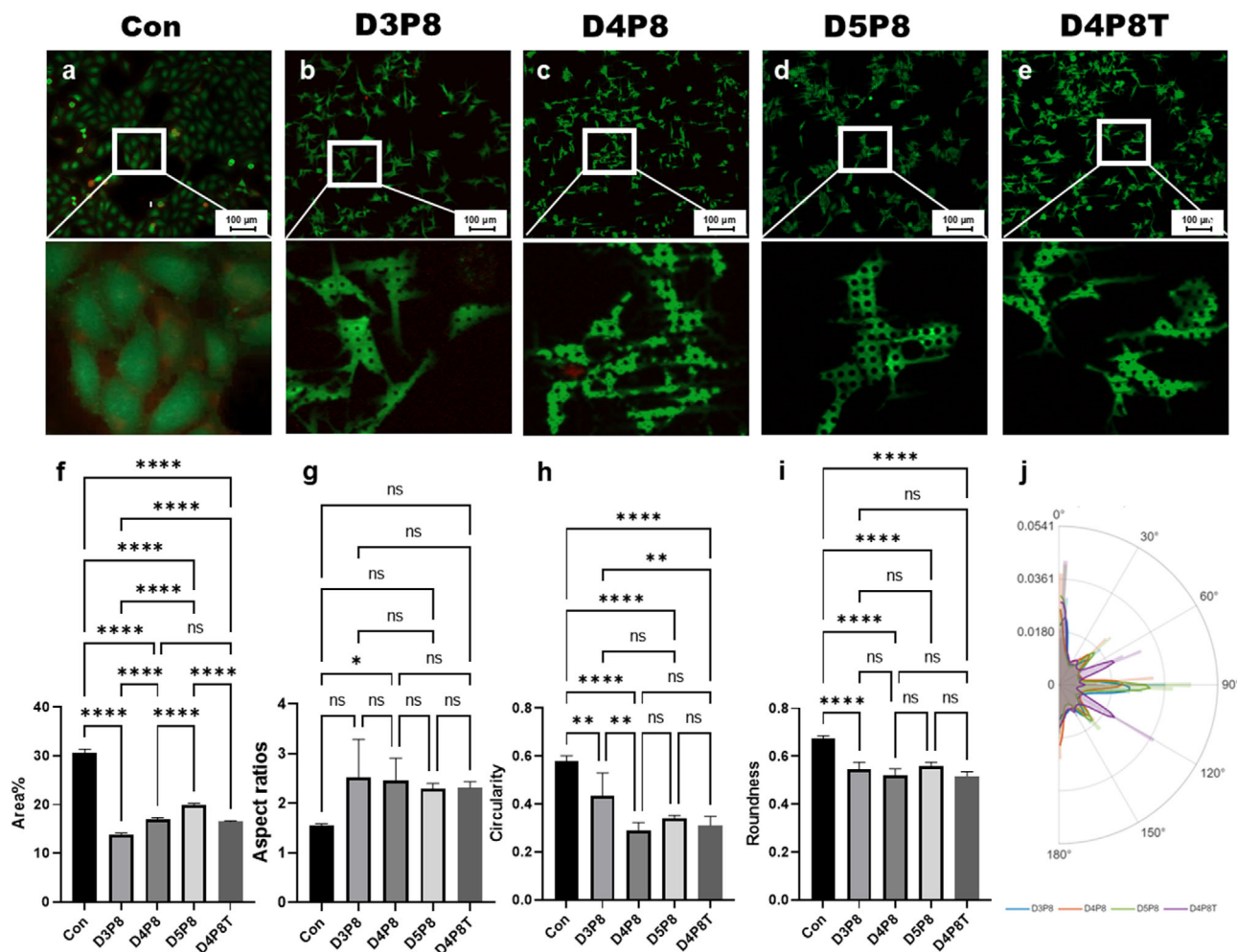


FIGURE 4 | Fluorescence images and morphological analysis of G292 cells cultured for 24 h on silicon chips. Cells were stained with a live/dead assay (green = live, red = dead) and imaged using an inverted fluorescence microscope (x100). (a) blank Si (Con), (b) D3P8, (c) D4P8 (d) D5P8, (e) D4P8T, (f) proportion of chip area occupied by cells on chips (area%), (g) aspect ratios of cells on chips, (h) circularity of cells on chips, (i) roundness of cells on chips, and (j) cell orientations. Bars indicate mean $\pm$ SEM. ns, not significant; * p < 0.05, ** p < 0.01, and **** p < 0.0001.

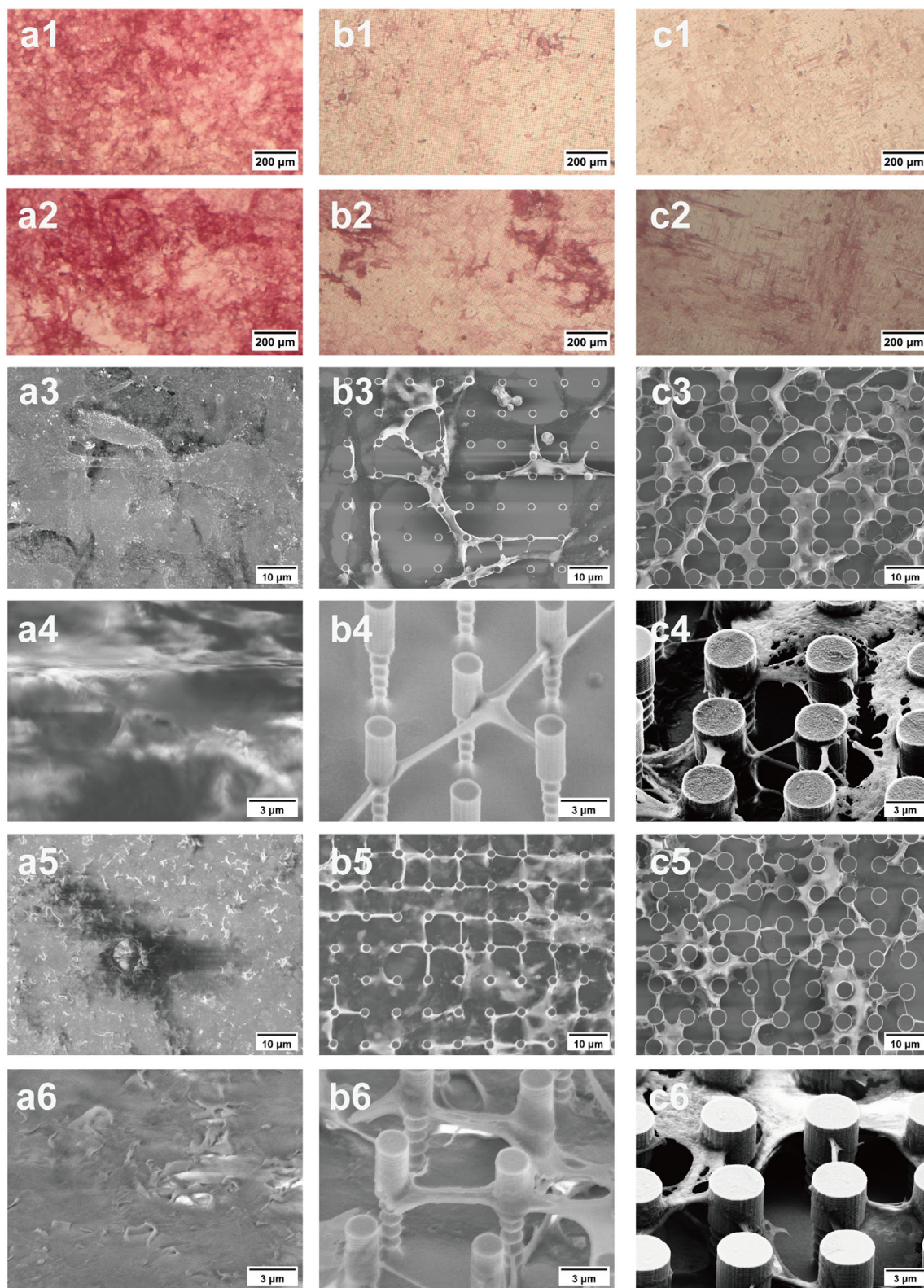


FIGURE 5 | Osteogenic differentiation of G292 cells cultured on wine glass-shaped silicon structured chips, compared to planar silicon substrates. (a1–c1, a2–c2) ALP staining after 7-day culture, (a) Con, (b) D3P8, (c) D5P8, 1: basal medium, 2: osteogenic medium. (a3–c3, a4–c4, a5–c5, and a6–c6) SEM images after ALP staining, (a) Con, (b) D3P8, (c) D5P8, 3: basal medium (top view), 4: basal medium (side view), 5: osteogenic medium (top view; $\times 1500$), 6: osteogenic medium (side view; $\times 6000$).

widely reported for cells cultured on microtopographically patterned substrates [40]. Conversely, cells on blank Si substrates displayed significantly higher circularity and roundness values (Figure 4g–h), reflecting a more symmetric spreading behavior

on the featureless flat surface. This observation is consistent with previous studies showing that cells spread isotropically on homogeneous planar substrates, whereas microtopographical cues reorganize cytoskeletal architecture and cell morphology [41].

Analysis of orientation distributions revealed clear geometry-dependent alignment patterns (Figure 4j). On the square micropatterns (D3P8, D4P8, and D5P8), cells predominantly aligned along the principal orthogonal axes, showing strong peaks at 0°, 90°, and 180°, with secondary but discernible peaks at 45° and 135° reflecting diagonal guidance cues inherent to the square lattice. In contrast, the triangular micropattern (D4P8T) produced a distinct threefold alignment signature, with major peaks at 0°, 60°, and 120°, consistent with its 60° rotational symmetry. Importantly, although D4P8 and D4P8T exhibited similar coverage and shape metrics due to comparable adhesive sidewall areas and identical pitch, their divergent orientation profiles highlight that lattice symmetry, rather than adhesion capacity, governs long-range cytoskeletal alignment. These findings suggest that local adhesive surface area and microscale spacing influence cell adhesion and spreading, while the geometrical symmetry of the scaffold architecture provides guidance cues that contribute to the global orientation of cells.

3.5.2 | Osteogenic Differentiation on Wine Glass-Shaped Microstructures

ALP staining was used to evaluate osteogenic differentiation of G292 cells after 7 days of induction. In the control group, cells already exhibited strong ALP activity under normal culture conditions, which was further enhanced following osteogenic induction, indicating a high baseline and inducible osteogenic potential. In contrast, cells cultured on the wine glass-shaped microstructures (D3P8 and D5P8) exhibited weaker ALP staining under standard conditions. However, after 7 days of osteogenic induction, ALP activity increased on both structured surfaces.

Semiquantitative analysis of ALP-positive staining area and relative staining intensity was performed to support image-based comparison and is provided in Figure S1. Although under osteogenic medium, D3P8 showed numerically higher values than D5P8 in the ALP-positive area and relative intensity, the differences were not statistically significant. Previous studies have suggested that inter-scaffold spacing can influence mesenchymal stem cells morphology, adhesion, and osteogenic marker deposition, with reported responses varying across the 1–6 μm spacing range [42, 43]. In the present study, D3P8 and D5P8 had estimated inter-scaffold spacings of ≈ 5 and 3 μm , respectively. Both designs therefore fall within a spacing range previously associated with microstructure-mediated cellular responses. Instead, the SEM observations suggested that cells on the wine glass-shaped microstructures exhibited mixed nonplanar adhesion (Figure 5). Cells appeared to interact with the scaffold necks, sidewalls, and inter-scaffold regions, with some cells showing bridging-like morphologies across adjacent structures. Together, these SEM observations suggest that the micro–nano structured surfaces may primarily influence cell adhesion configuration, morphology, and spatial organization through geometry-dependent cues. This interpretation is supported by previous studies showing that osteoblasts can sense microstructure structures through changes in adhesion organization, intracellular signaling, and cytoskeletal behavior [44, 45]. In addition, anisotropic microstructure arrays have been reported to guide cells into inter-scaffold spaces and promote pronounced cell elongation and directional alignment [46]. Further quantitative assays are required to determine whether these

structure-mediated cellular responses directly contribute to enhanced osteogenic differentiation.

4 | Conclusion

Compared to conventional micro–nanofabrication techniques such as electroplating, atomic layer deposition, and two-photon polymerization, the Bosch DRIE process developed in this study uniquely combines scalability, time efficiency, and high structural controllability. This makes it particularly advantageous for fabricating 3D silicon microstructures with complex topographies. By systematically tuning etching parameters such as high-frequency power, etch duration, and etch–deposition loop design, three distinct 3D topographies, obelisk, scalloped pillars, and wine-glass-shaped scaffolds, were successfully fabricated with precise control over height (0.2–13.3 μm), sidewall angle (72°–77°), sharpness, and overall morphology.

The process was implemented on 4-inch silicon wafers, demonstrating wafer-scale compatibility and time efficiency, with each etching cycle completed in under 5 min. The resulting structures exhibit submicron-scale resolution, high aspect ratios, and well-defined profiles, confirming the platform's capability to achieve complex geometries with excellent reproducibility. Moreover, all structures are composed of pure silicon, ensuring compatibility with subsequent microfabrication processes such as metallization, photolithography, and surface functionalization.

Besides, wine glass-shaped 3D microstructure architectures governed cell coverage, elongation, and alignment through the combined effects of sidewall-dominated adhesion, gap-dependent spanning, and lattice symmetry, while selected designs partially recovered osteogenic activity under inductive conditions. Beyond its immediate structural versatility, this work expands the application scope of the Bosch DRIE process from conventional Micro-Electro-Mechanical Systems manufacturing to micro–nano topographies relevant for biointerface and tissue engineering. The demonstrated platform holds promise for future integration into advanced microsystems, including neural interfaces, biosensors, and high-density microelectrode arrays.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.