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Scalable Flow Reactors for Stable Biofilm Formation and Continuous Whole-Cell Catalysis

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ABSTRACT

Stable and productive biofilms are highly attractive for continuous whole-cell biocatalysis, yet their controlled formation and long-term operation remain challenging. Here, we introduce a modular and scalable microfluidic reactor platform that exploits flow-induced deposition to generate robust biofilms directly from suspended cells. Starting from a validated pillar reactor design, we systematically scale reactor geometries and apply computational flow simulations to identify microscale hydrodynamic features that govern cell attachment, streamer formation, and biofilm stability. Fluorescence microscopy reveals the emergence of stratified biofilm architectures enriched in extracellular DNA that persist under continuous flow. Enzymatic depletion of extracellular DNA selectively delays early streamer formation, highlighting its critical role during initial matrix assembly. Under continuous flow, biofilm growth and shear-induced detachment establish a dynamic steady state that maintains a self-sustaining catalytic matrix, enabling long-term continuous whole-cell biocatalysis in an upscaled reactor where the deposited biofilms remain catalytically active and structurally stable for at least 28 days. Together, these results establish flow-structured microreactors as a generalizable strategy for hydrodynamically controlled biofilm engineering, linking microscale flow fields to biofilm architecture, long-term stability, and catalytic function, and providing a foundation for scalable continuous whole-cell biocatalysis.

1 | Introduction

The transition toward a sustainable chemical industry increasingly relies on innovative biotechnological solutions that can complement or replace traditional petrochemical processes [1–3]. Whole-cell biocatalysis and microbial fermentation play a central role in this development, as they provide access to a bio-based circular economy by enabling environmentally compatible and economically viable production routes [4–7]. Despite these advantages, further technological progress is required to overcome limitations in process efficiency, catalyst stability, and operational robustness.

One promising strategy to address these challenges is the immobilization of biocatalysts in compartmentalized flow systems. Inspired by the advances of flow chemistry in synthetic organic chemistry, efforts in white biotechnology increasingly focus on transferring modular, machine-operated synthesis to continuous, miniaturized biocatalytic reactors [2, 8–17]. Microreactors offer unique physicochemical advantages due to their small characteristic dimensions, including high surface-to-volume ratios, efficient heat transfer, short diffusion distances, and highly controlled laminar flow profiles. These features facilitate improved mass transport between compartments and enable streamlined integration of substrate supply, product removal, and multi-step

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cascade reactions while minimizing unwanted cross-reactivity. Additionally, microreactor platforms provide inherent compatibility with automation, in-line analytics, purification, and downstream processing strategies, supporting the development of end-to-end continuous biomanufacturing workflows. Beyond these process advantages, microfluidic reactors enable precise control over hydrodynamic microenvironments at the micrometer scale [18]. This capability allows defined shear fields and flow structures to be engineered within the reactor, providing a powerful platform to investigate how hydrodynamics influence microbial attachment, biofilm formation and the organization of living catalytic materials under continuous flow conditions, while maintaining optical accessibility for real-time observation.

Whole cells are particularly attractive for implementation in continuous flow biocatalysis. Their low-cost production, intrinsic cofactor regeneration, and protective intracellular environment often render them more robust than isolated enzymes. As a consequence, flow-based whole-cell biotransformations are increasingly explored as high-efficiency systems capable of combining multiple reaction steps into a single continuous operational unit [19]. While fixed-bed reactors with chemically or physically immobilized microorganisms represent established approaches [20–22], the use of biofilms as self-generated immobilization matrices remains comparatively underdeveloped [23]. For industrial microorganisms with limited intrinsic biofilm-forming capability, new strategies are required to improve cell retention and long-term stability, potentially through tailored reactor geometries, engineered biofilm formation, or metabolic optimization.

A recent study from our group demonstrated that microfluidic reactor geometries can be deliberately exploited to induce flow-driven deposition of biomaterials, including recombinant *E. coli*, under well-defined hydrodynamic conditions [24]. In this system, flow-assisted cell aggregation resulted in the formation of stable living materials directly within the reactor, enabling unprecedented volumetric productivities exceeding $3 \text{ kg L}^{-1} \text{ d}^{-1}$ that remained constant over several days. Notably, these productive biofilms could be operated for more than ten days, underscoring the transformative potential of flow-induced immobilization for continuous whole-cell biocatalysis. These findings suggest that controlled biofilm formation in microfluidic environments represents an unexplored design principle for advanced bioreactor engineering.

Building on this foundation, the present study aims to refine and systematically investigate this new class of flow-assisted microfluidic bioreactors. Using recombinant *E. coli* strains that express fluorescent or biocatalytically relevant proteins, we analyze how reactor size scaling affects flow-induced biomass deposition and whether staining-based methods under continuous flow can provide mechanistic insight into the morphological evolution and long-term behavior of the resulting living materials. A central question guiding this study is whether the deposition mechanism observed in the reactors represents a natural form of surface immobilization, that is, genuine biofilm formation, rather than a purely physicochemical accumulation process. By integrating whole-cell biocatalysis with microfluidic flow engineering, this work seeks to deepen the fundamental understanding of flow-mediated biomass attachment and to identify strategies

for enabling robust, scalable and highly productive continuous bioprocesses. The conceptual framework of hydrodynamically structured biofilm deposition investigated in this work is illustrated in Figure 1A, highlighting how defined flow fields around the pillar geometry promote localized cell attachment, streamer formation and the development of stable biofilm structures under continuous flow.

2 | Results and Discussion

To establish a suitable platform for studying flow-induced biofilm formation, we first validated the deposition behavior of recombinant *E. coli* in the original pillar reactor and subsequently developed a series of scaled reactor geometries to improve manufacturability and experimental robustness. After confirming that the upscaled reactor supported reliable biomass deposition, this geometry was selected for subsequent biofilm analyses.

2.1 | Establishing a Scalable Microfluidic Reactor Platform

To verify that the reactor platform used in this study reproduces the behavior reported by Lemke et al. [24], we first re-established the characteristic flow-induced deposition of *E. coli* in the pentagonal pillar reactor. In the original work, the pillar geometry was shown to generate localized regions of elevated shear stress and vorticity that coincide with preferential cell attachment and the formation of streamline-oriented filamentous structures. While shear stress has been implicated in biofilm formation, potentially through shear-induced metabolic or physiological responses [25], the dominant mechanism underlying biomass deposition in this reactor remains unresolved. In particular, it is unclear whether deposition is driven primarily by shear-mediated changes in cell adhesion, by prolonged local residence times associated with velocity gradients and recirculation zones, or by a combination of both effects. Regardless of the precise contribution of these mechanisms, the presence of hydrodynamically defined shear and vorticity patterns is clearly essential for robust biomass accumulation and thus constitutes the mechanistic foundation of flow-induced immobilization in this system.

To experimentally assess these hydrodynamically driven deposition phenomena, we employed the microfluidic setup (Figures S1 and S2) previously established introduced by Lemke et al. [24]. The system consists of a microfluidic chip containing three identical pillar reactors mounted between PMMA support and connector plates and connected to syringe pumps via PTFE tubing and flangeless fittings. Bacterial suspensions were perfused through the reactors under controlled flow conditions, and the effluent was collected in a waste container, while biomass deposition was monitored in real time by fluorescence microscopy. Using this setup and the original reactor geometry, referred to here as R1_20, an *E. coli* eGFP suspension adjusted to $\text{OD}_{600} = 0.05$ was injected at a flow rate of $4 \mu\text{L min}^{-1}$ and imaged over a period of 12 h.

As expected, the cells accumulated at the pillar surfaces, forming visible biomass deposits that extended downstream into filamentous, streamline-aligned structures. These features match

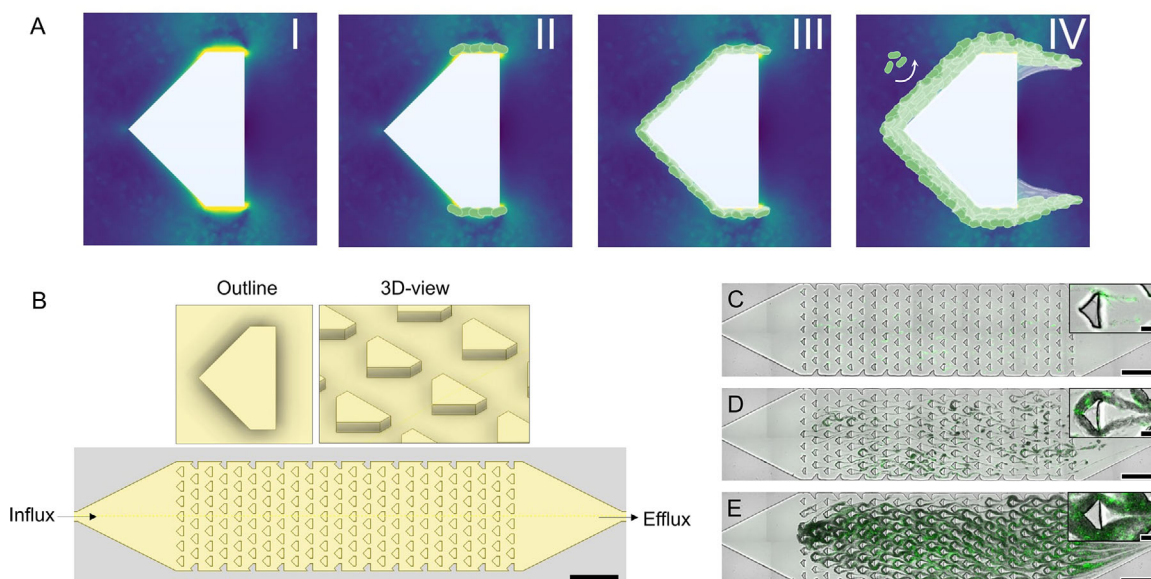


FIGURE 1 | Deposition of *E. coli* cells in a microfluidic deposition reactor. (A) Conceptual illustration of hydrodynamically structured biofilm formation. Defined flow fields generated by the pillar geometry (I) create localized hydrodynamic niches that promote initial cell attachment (II). Subsequently, attached cells form early biofilm layers on the pillar surface (III), which develop into mature biofilms with streamer formation (IV). Under continuous flow, biofilm growth and shear-induced detachment establish a dynamic steady state that prevents excessive accumulation, enables removal of detached biomass, and supports long-term stable biofilm operation. (B) Schematic illustration of the microfluidic reactor architecture, highlighting the pentagonal pillar geometry that structures the flow field. A 3D view of the reactor is shown to illustrate the overall channel layout. Scale bar = 500 μm ; pillar insets are not drawn to scale. (C–E) Time-resolved flow-induced deposition of *E. coli* expressing eGFP after (C) 2 h, (D) 8 h and (E) 12 h. The images illustrate the gradual accumulation of biomass and the formation of extended, streamline-oriented structures (streamers). Cell suspensions were injected at a flow rate of 4 $\mu\text{L} \cdot \text{min}^{-1}$. Bright-field images are shown as overlays with the corresponding eGFP fluorescence signals ($\lambda_{\text{exc}} \sim 488 \text{ nm}$, $\lambda_{\text{em}} \sim 507 \text{ nm}$) to visualize the spatial distribution of the deposited biomass. Flow direction is from left to right. Reactor type: R1_20. Scale bars are 500 μm (overview) and 50 μm (inset).

the previously reported flow-induced streamer formation and confirm that the reactor reproduces the characteristic deposition behavior of recombinant *E. coli* under the conditions used here. The temporal development of these structures is shown in Figure 1. The observed filamentous extensions originate from anchored biomass at the pillar edges and grow in the direction of flow, which is fully consistent with the behavior described for *E. coli* in the original study [24].

From these initial observations, we conclude that both the reactor geometry and the microbial system provide a suitable and well-defined basis for the subsequent experiments presented in this work. The filamentous structures are most likely streamers, defined as filamentous bacterial biofilms formed in flowing systems [26]. Streamers are known to represent highly elastic and mechanically robust biofilm architectures whose backbone consists largely of extracellular DNA (eDNA), as demonstrated for *Pseudomonas aeruginosa* [26]. Their reproducible formation in the reactor system therefore suggests that the deposited *E. coli* biomass does not merely accumulate passively but establishes a structurally organized, shear-resilient matrix that can persist under continuous flow and be interrogated in the following sections.

After confirming that the original pillar reactor reliably reproduced the expected flow-induced deposition behavior of recombinant *E. coli*, we next addressed a major practical limitation of the initial design. As reported previously [24], fabrication

of the microstructured PDMS reactors by double casting was associated with a high rejection rate and frequent clogging during operation. These limitations primarily arose from the delicate pillar geometry in combination with the small channel height of the original reactor R1_20, where the index denotes a channel height of 20 μm corresponding to the pillar height. To improve manufacturability, reproducibility, and operational robustness, and to enable extended cultivation periods, we therefore developed a series of linearly scaled reactor geometries. An overview of the scaling strategy, including the geometric enlargement of the reactor structures, the resulting changes in cross-sectional area, and representative hydrodynamic features obtained from CFD simulations, is shown in Figure 2 and Figure S3.

During scaling, the overall footprint of the machined master (76 mm $\times$ 26 mm), accommodating three individual flow-deposition reactors, was retained. Depending on the scaling factor, the total lengths of the inlet and outlet channel sections ranged from approximately 10 to 26 mm, while channel widths were increased linearly from 1.1 mm (R1) to 2.2 mm (R2) and 4.4 mm (R4). The pentagonal pillar geometry of the original R1_20 design, featuring lateral pillar dimensions of 100 μm and inter-pillar gaps of 50 μm along the Y direction, served as the template for systematic geometric scaling. As illustrated in Figure 2 and Figure S3, linear scaling of the lateral X and Y dimensions by factors of two and four yielded the reactor families R2_Z and R4_Z, respectively, where Z denotes the channel height in micrometers. Independent variation of the channel height

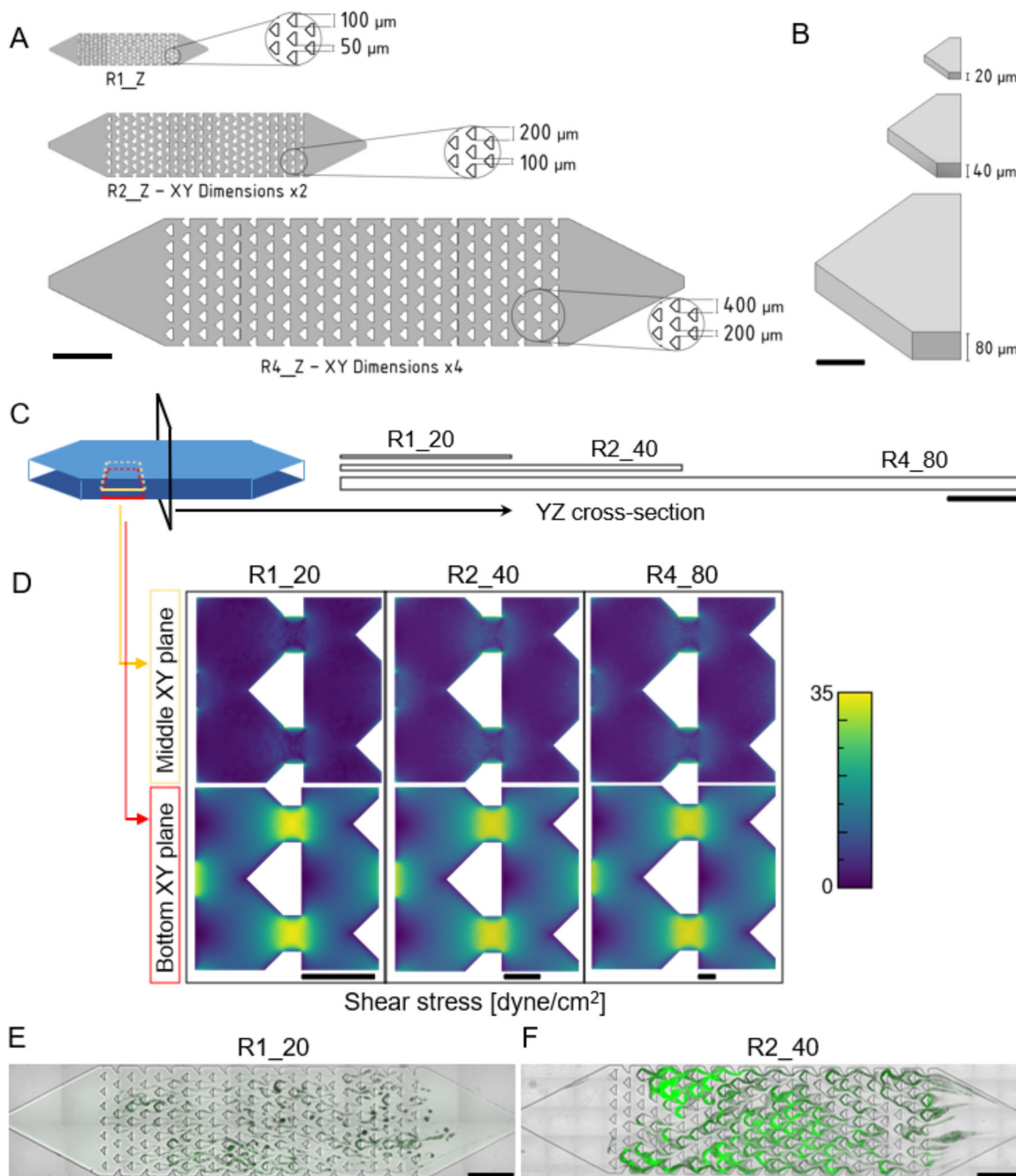


FIGURE 2 | Reactor scaling strategy and preservation of hydrodynamic features. (A, B) Schematic illustration of the geometric upscaling of the original reactor R1_20, showing the doubling and quadrupling of all lateral dimensions to generate the reactor families R2_Z and R4_Z, respectively, where Z denotes the channel height in micrometers. Reactors are shown schematically and drawn to scale. Scale bars = 2 mm (A) and 100 μm (B). (C) Cross-sectional areas of selected reactor geometries. Doubling and quadrupling the lateral dimensions increase the channel cross-sectional area by factors of 4 and 16, respectively, necessitating proportional adjustment of the volumetric flow rate to maintain comparable flow velocities or shear conditions. Scale bar = 500 μm. (D) Representative shear stress maps obtained from CFD simulations of reactors R1_20, R2_40 and R4_80, shown either at the channel mid-height or at the bottom wall plane, demonstrating that characteristic shear stress hotspots at the pillar flanks at mid-height and between adjacent flanks near the bottom wall are preserved when increasing the flow rate from 4 to 32 μL min⁻¹ for R2_40 and to 256 μL min⁻¹ for R4_80. (E, F) Fluorescence microscopy images ($\lambda_{\text{exc}} \sim 488$ nm, $\lambda_{\text{em}} \sim 507$ nm) demonstrating reliable biomass deposition in reactors R1_20 and R2_40 after 8 h of cell perfusion, confirming the robustness of the deposition behavior and the suitability of R2_40 as the standard reactor geometry for subsequent experiments. Scale bars: E = 500 μm and F = 1 mm.

resulted in the full set of reactor geometries R2_40, R2_80, R2_120 and R4_80, R4_160, R4_240. Importantly, the established soft-lithographic fabrication workflow, including PDMS double casting and plasma bonding to glass substrates, remained fully compatible with all scaled designs.

To quantify how geometric upscaling influences the flow field, we performed extensive CFD simulations across all reactor geometries, focusing on flow velocity, shear stress, and vorticity. Simulations of the enlarged reactors confirmed that the overall structure of the velocity field, including stagnation zones and high-velocity regions, was preserved relative to the original R1_20 geometry (Figure S4). Cross-sectional analyses of the R1_20 reference reactor revealed peak flow velocities between adjacent pillar flanks along the Y direction in the mid-height plane, where local flow velocity is maximal (Figure S5). Elevated shear stress was primarily localized at the pillar flanks and within the narrow constrictions between adjacent pillars near the upper and lower channel walls, regions previously identified as preferential nucleation sites for biomass deposition [24]. Equivalent analyses were performed for the scaled geometries R2_40 and R4_80 (Figure S6). As a consequence of this velocity-preserving scaling strategy, the mean residence times in the deposition region remain within a similar range across the reactor families despite the substantial increase in reactor dimensions (Table S1), indicating that comparable hydrodynamic exposure times are maintained during scaling.

Because doubling and quadrupling the lateral dimensions increase the channel cross-sectional area by factors of 4 and 16, respectively, the volumetric flow rate was increased from $4 \mu\text{L min}^{-1}$ in R1_20 to $16 \mu\text{L min}^{-1}$ for R2_40 and to $64 \mu\text{L min}^{-1}$ for R4_80 in order to maintain comparable mean flow velocities (Figure S6). While this approach successfully reproduced the global velocity field of the original reactor, the absolute shear stress levels in deposition-relevant regions were reduced, reflecting the dependence of shear stress on both flow velocity and characteristic channel dimensions. Nevertheless, the qualitative structure of the shear stress field, including the spatial localization of high-stress regions at pillar flanks and constrictions, was preserved across all geometries. According to scaling relations, achieving shear stress magnitudes comparable to those of R1_20 would require substantially higher flow rates, specifically $32 \mu\text{L min}^{-1}$ for R2_40 and $256 \mu\text{L min}^{-1}$ for R4_80 (Figure 2D; Figure S6). Given experimental constraints and the unresolved question of whether local shear stress or flow velocity is the dominant parameter governing biomass deposition, we therefore selected flow conditions that preserved comparable velocity fields rather than absolute shear stress values. Additional simulations addressing the influence of channel height on flow velocity, shear stress, and vorticity are shown in Figures S7–S9, revealing a systematic decrease in shear stress intensity with increasing channel height when flow rates are adjusted only to maintain flow velocity.

Experimental evaluation of biomass deposition across the different reactor geometries demonstrated that reliable and reproducible biofilm formation could be achieved in the majority of designs. Among these, reactor R2_40 provided particularly favorable optical accessibility for fluorescence microscopy (Figure 2E), in combination with substantially improved fabrication yield and

reduced clogging during operation. Based on the combined criteria of manufacturability, mechanical robustness, hydrodynamic similarity to the original design, and experimentally validated deposition performance across the scaled reactor family, R2_40 was therefore selected as the standard reactor geometry for all subsequent studies addressing biofilm formation, structural organization, and dynamic behavior under flow. In contrast, long-term whole-cell biocatalysis experiments were conducted in the further upscaled reactor R4_160, which enables substantially higher volumetric flow rates and throughput than R2_40 while preserving the deposition-relevant hydrodynamic features identified in the smaller-scale reactors. This separation allowed mechanistic investigations to be performed under conditions optimized for imaging and control, while functional biocatalysis was evaluated under flow regimes more representative of preparative operation.

For the R4_160 reactor, flow conditions were deliberately selected to first promote efficient biomass deposition and subsequently maintain quasi-steady catalytic operation. During the initial deposition phase, cell suspensions were perfused at a flow rate of $128 \mu\text{L min}^{-1}$, corresponding to a mean residence time of approximately 5 s within the deposition region of the reactor. For the subsequent biocatalytic operation, the flow rate was reduced to $35 \mu\text{L min}^{-1}$, resulting in a residence time of approximately 18 s. These short residence times are characteristic of microfluidic flow systems and ensure continuous substrate supply and product removal at the biofilm interface during steady-state catalysis.

2.2 | Early-Stage Biofilm Formation and Matrix Organization Under Flow

To assess whether the biomass deposited under flow conditions in the R2_40 reactor constitutes a bona fide biofilm, we examined the extracellular matrix during the initial phase of deposition by applying a DNA-specific staining procedure. Propidium iodide (PI) was chosen because it binds strongly to DNA and thereby enables visualization of extracellular DNA (eDNA), a key structural component of bacterial biofilms. Although PI preferentially stains dead or membrane-compromised cells and may underestimate the viability of adherent bacteria [27], the method offers two advantages relevant for the present analysis: (i) PI can serve as a reliable indicator for the presence and spatial distribution of eDNA in the extracellular matrix, and (ii) PI-bound DNA exhibits a red-shifted fluorescence emission, allowing unambiguous identification of DNA-rich regions when imaged alongside eGFP-expressing cells. Because eDNA is known to arise both through controlled autolysis and through stochastic cell lysis events during biofilm development [28], the appearance of PI-positive structures provides mechanistic insight into the early formation and organization of the matrix.

To visualize cell deposition and matrix development simultaneously, flow experiments were performed in which an *E. coli* eGFP suspension adjusted to $\text{OD}_{600} = 0.05$ supplemented with PI was injected into the R2_40 reactor at a flow rate of $16 \mu\text{L min}^{-1}$. Two complementary imaging modalities were employed: fluorescence microscopy for mesoscale visualization of streamer formation (Figure 3A–E) and confocal laser-scanning microscopy (CLSM) for high-resolution structural analysis (Figure 3F,G). After 8 h

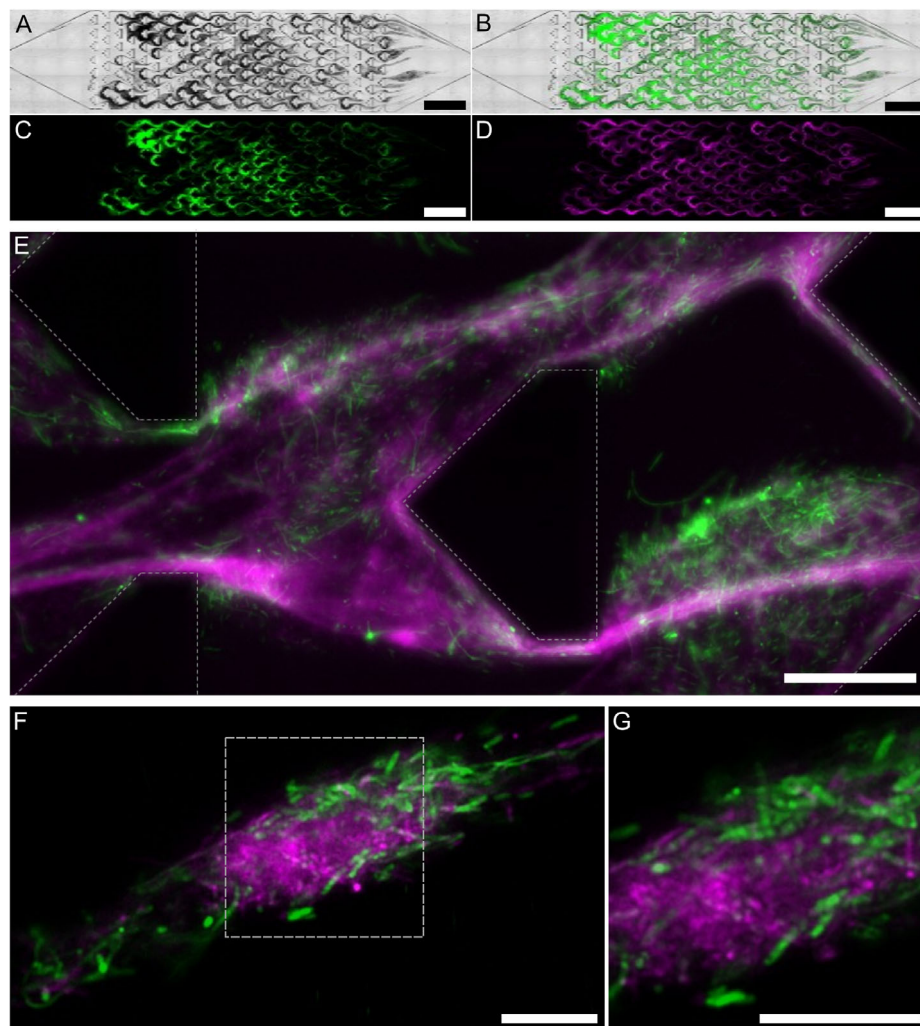


FIGURE 3 | Morphology of early-stage *E. coli* eGFP deposition during simultaneous PI staining in a microfluidic pillar reactor. (A–D) Bright-field (A), BF + GFP overlay (B), GFP ($\lambda_{\text{exc}} \sim 488$ nm, $\lambda_{\text{em}} \sim 507$ nm) (C) and PI ($\lambda_{\text{exc}} \sim 535$ nm, $\lambda_{\text{em}} \sim 617$ nm) (D) images after 8 h of deposition at $16 \mu\text{L min}^{-1}$ reveal streamer formation and co-localization of cellular biomass with PI fluorescence. (E) Higher-magnification fluorescence image after 2 h 15 min showing filamentous, web-like matrix structures associated with the deposited cells. (F,G) High-resolution LSM images acquired after 3 h 30 min highlighting the spatial proximity of eGFP-labelled cells and PI-positive regions within compact streamer structures. Flow direction from left to right. Reactor: R2_40. Scale bars: A–D = 1 mm; E = 100 μm ; F,G = 20 μm .

of deposition, distinct streamer-like structures spanning multiple pillars were observed in bright-field, eGFP and PI channels (Figure 3A–D). The strong spatial correspondence between eGFP and PI signals indicates that PI fluorescence arises predominantly in regions densely populated with cells. At higher magnification, the deposited biomass appeared embedded within a web-like, filamentous matrix (Figure 3E), consistent with early-stage streamer architectures. Notably, these structures show morphological similarity to filamentous networks previously observed during the immobilization of polymeric hydrogels composed of single-stranded DNA generated in situ by enzymatic rolling-circle amplification [24]. While this comparison does not constitute direct proof of composition, it supports the interpretation that the PI-positive structures observed here are associated with DNA-rich matrix components.

High-resolution CLSM imaging provided further insight into the fine-scale organization of the deposited biomass (Figure 3F,G). PI fluorescence was closely associated with the cellular network,

indicating the presence of DNA-rich regions both within and surrounding cell aggregates. In addition, PI-positive features with morphologies resembling cellular remnants or partially lysed cells were occasionally observed, suggesting that a fraction of the detectable DNA may originate from lysis events occurring during early deposition. Such observations are consistent with established models of biofilm development, in which extracellular DNA released through cell lysis contributes to matrix assembly and mechanical cohesion [29]. Although these data do not allow definitive conclusions regarding the origin or specific function of the DNA-rich material, the combined presence of diffuse PI-positive matrix-like structures and their spatial association with eGFP-labelled cells supports the interpretation that the deposited biomass exhibits characteristic features of early biofilm formation rather than representing purely physical cell aggregation.

To investigate how these early matrix-associated structures evolve during prolonged cultivation under flow, we next performed long-term growth experiments followed by PI staining. The aim of

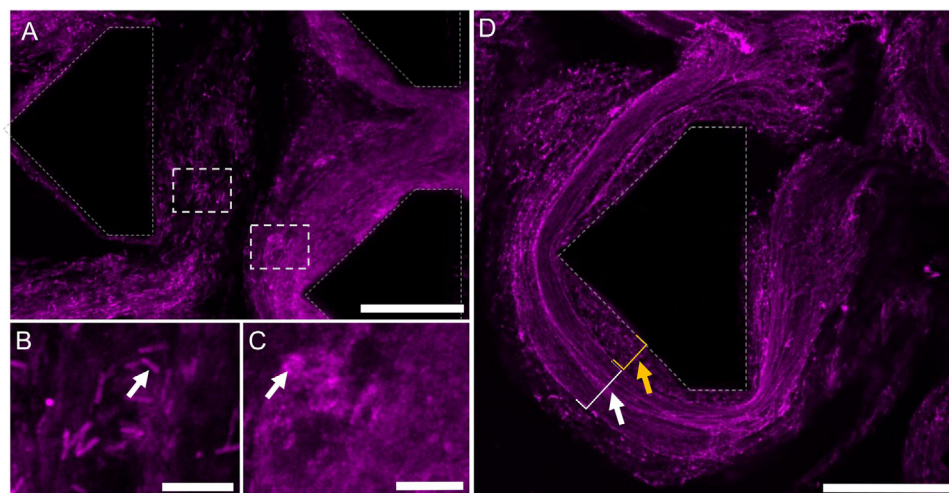


FIGURE 4 | Morphology of the biofilm matrix after 6 days of continuous operation in a microfluidic pillar reactor. An *E. coli* cell suspension was injected into reactor R2_40 for 4 h at $16 \mu\text{L min}^{-1}$ to initiate colonization, followed by cultivation under ampicillin-containing LB medium at $4 \mu\text{L min}^{-1}$ for 6 days. The mature biofilm was subsequently stained with PI ($c_{\text{PI}} = 4 \mu\text{g mL}^{-1}$). (A–D) Fluorescence ($\lambda_{\text{exc}} \sim 535 \text{ nm}$, $\lambda_{\text{em}} \sim 617 \text{ nm}$) and bright-field images acquired within the first 3 h of staining. Two characteristic PI-associated morphologies were observed: (B, white arrow) cellular PI signals and (C, white arrow) diffuse PI-positive regions indicative of eDNA-rich matrix. (D) Typical biofilm architecture at a pillar surface, showing a dense, network-like inner layer (yellow arrow) and a filamentous, flow-oriented outer layer (white arrow). Flow direction from left to right. Reactor: R2_40. Scale bars: A+D = $100 \mu\text{m}$; B+C = $20 \mu\text{m}$.

this analysis was to determine whether the initially deposited biomass develops into a structurally consolidated biofilm architecture and to identify morphological changes that emerge during maturation. Because PI binds strongly to DNA and enables visualization of eDNA-rich regions within the extracellular matrix, its application provides insight into matrix composition and spatial organization in later-stage biofilms.

To enable extended cultivation without detaching the developing biofilm, the experimental setup was modified to a modular flow system incorporating a three-way valve configuration (Figure S10B). After an initial deposition phase of 4 h, during which *E. coli* eGFP cells were perfused through the R2_40 reactor at $16 \mu\text{L min}^{-1}$, the flow was switched to ampicillin-supplemented LB medium and reduced to $4 \mu\text{L min}^{-1}$. A tenfold higher starting cell density ($\text{OD}_{600} = 0.5$) was used to accelerate colonization. Biofilms were cultivated for up to 12 days in two independent reactor replicates, after which PI staining was applied for 3 h after 6 days and for 4.5 h after 12 days to visualize matrix organization (Figure 4; Figures S11–S13).

After 6 days of cultivation, the biofilm displayed a consolidated and clearly stratified architecture (Figure 4). Strong PI fluorescence emerged rapidly, indicating substantial DNA content within the matrix. Two characteristic PI-associated morphologies were consistently observed: PI signals with cellular outlines (Figure 4B) and diffuse PI fluorescence consistent with eDNA-rich matrix components (Figure 4C). These two populations likely reflect multiple DNA sources within the biofilm, consistent with both active eDNA release and cell lysis mechanisms described in the literature [28, 29].

At the pillar surfaces, the biofilm exhibited pronounced spatial organization (Figure 4D). The pillar-adjacent basal layer displayed a compact, network-like morphology with comparatively

weak PI staining, whereas the outer, flow-facing layer formed filamentous structures with strong PI signals. A representative example of this layered morphology is shown in Figure S13, where PI-positive outer structures contrast with deeper, weakly stained regions (white and yellow arrows, respectively). Time-resolved PI imaging (Figure S11) further supported this stratification, as PI fluorescence first appeared in the outer layer and only later permeated into the inner matrix. This pattern suggests that the outer region experiences greater hydrodynamic stress and potentially higher matrix turnover, while the deeper layer forms a more protected, mechanically sheltered zone that supports long-term structural integrity.

Live-cell imaging revealed additional dynamic processes that may contribute to the formation of the outer filamentous layer. Streamers frequently detached from upstream pillars and reattached downstream, elongating along local flow fields. This detachment and redeposition process, together with prevailing hydrodynamic forces, may contribute to the streamlined architecture observed in mature biofilms. Although streamers are mechanically robust, they do not constitute stable long-term structures; instead, their recurrent formation may facilitate surface colonization and biomass redistribution in flowing systems. The repeated occurrence of this behavior suggests that it represents a conserved architectural motif, potentially modulated through regulatory mechanisms such as quorum sensing [29, 30]. Taken together, these observations suggest that biomass accumulation in the reactor reflects a dynamic balance between attachment, growth and shear-induced removal under continuous flow, as illustrated in Figure 1A. Stable biomass retention is supported by the reactor geometry, which provides pillar-associated anchoring sites within localized hydrodynamic niches, and by matrix-mediated cohesion within the developing biofilm. Rather than forming static structures, the deposited biofilms therefore behave as dynamic systems in which biomass is continuously

redistributed through detachment and redeposition processes. Such a dynamic steady state likely prevents excessive biomass accumulation while maintaining sufficient surface-associated biomass to sustain biofilm development and function under flow conditions.

Across experiments, biological replicates frequently developed markedly different large-scale morphologies. The representative example in Figure S11 illustrates that one reactor exhibited extended PI-positive regions, whereas the second showed predominantly cellular PI signals. These differences highlight how subtle variations in local hydrodynamic conditions or transient clogging events can influence the trajectory of biofilm development. Nevertheless, both replicates consistently displayed the layered organization with filamentous, PI-accessible outer regions.

Staining of 12-day-old biofilms (Figure S12) confirmed that mature matrices can be deeply infiltrated by PI when sufficient exposure time is provided. Notably, prolonged decolorization for up to 17 h 30 min did not fully remove PI from the matrix, demonstrating a strong interaction between PI, cellular material and the eDNA-rich scaffold, even though continuous perfusion promotes gradual diffusive loss of the intercalating dye.

To further examine the reversibility of PI staining, mature biofilms were subjected to consecutive PI pulses in two independent reactor experiments (Figures S14 and S15). After each overnight rinsing step, PI fluorescence nearly disappeared, and subsequent staining pulses produced progressively weaker signals. Although differences in deposition durations, staining times and imaging settings limit quantitative interpretation, the overall trend confirms that PI staining is transient under continuous flow and not suitable for repeated labeling of the same biofilm. Nevertheless, these pulse experiments reinforce the use of PI as a sensitive probe for matrix accessibility during individual staining cycles.

Taken together, these findings show that *E. coli* biofilms cultivated under continuous flow develop stratified architectures with distinct spatial and functional characteristics. The outer layer is filamentous and highly accessible to PI staining under flow, whereas the deeper layer forms a compact matrix that remains largely unstained during early PI exposure and likely contributes to long-term structural stability. Although the available imaging is limited to two dimensions, the recurring spatial patterns strongly indicate a layered biofilm architecture that emerges from the interplay of eDNA accumulation, hydrodynamics, cell lysis, motility-driven restructuring [31] and rheotactic responses to flow [32].

Beyond these specific observations, it is important to note that the molecular organization and stabilization of extracellular DNA within biofilm matrices is currently the subject of intense investigation. Recent studies have revealed that eDNA can adopt higher-order architectures, including lattice-like arrangements related to Holliday junction recombination intermediates [33], as well as non-canonical secondary structures such as G-quadruplexes that contribute to the viscoelastic properties of biofilm matrices [34]. In this context, the high-resolution imaging of eDNA-associated structures presented here, obtained directly within a microflu-

idic flow environment, provides a complementary experimental perspective. While the present data do not resolve the molecular conformation of eDNA, the observed spatial organization and accessibility patterns suggest that microfluidic reactors may offer a powerful platform to visualize and interrogate eDNA matrix architectures under well-defined hydrodynamic conditions.

2.3 | Role of Extracellular DNA in Initial Deposition and Streamer Formation

The identification of extracellular DNA within the deposited biomass raised the question of whether eDNA plays an essential role in the initial deposition process and the formation of early biofilm structures in the microfluidic reactor. To address this, we examined the effect of DNase I on cell attachment, biofilm initiation and streamer development under flow (Figure 5). DNase I enzymatically degrades extracellular DNA and is widely used to probe the structural contribution of eDNA to biofilm formation and mechanical stability [35, 36]. Importantly, a comprehensive review by Okshevsky et al. [37], highlights that the impact of DNase I on biofilms is highly species- and context-dependent. While DNase I has been shown to disperse established biofilms of several organisms, including *E. coli*, it is often less effective in preventing initial biofilm formation. The present study therefore specifically addresses how enzymatic depletion of eDNA affects the very early stages of deposition and streamer formation under defined microfluidic flow conditions.

To assess the influence of eDNA depletion on deposition dynamics, two *E. coli* eGFP suspensions adjusted to $OD_{600} = 0.05$ containing PI were prepared in parallel. One served as an untreated control, whereas the second was supplemented with DNase I at twice the concentration used in earlier studies to maximize the enzymatic effect [26]. Both suspensions were injected simultaneously into separate microfluidic reactors under identical flow conditions ($16 \mu\text{L min}^{-1}$), and biofilm development was monitored by time-lapse fluorescence microscopy over 8 h (Figure 5; Figures S16–S18).

In the untreated culture, streamer structures emerged within the first hour (Figures S16–S18), consistent with the rapid colonization behavior observed in previous experiments. In contrast, the DNase-treated sample exhibited almost no detectable biomass deposition during the first 6 h of perfusion (Figure 5C, lower row). Quantitative analysis of reactor coverage confirmed that DNase treatment caused a pronounced delay in deposition, with both biomass coverage and PI fluorescence remaining at low levels until approximately 6 h (Figure 5A,B). After this delay, biomass accumulation increased and eventually approached levels comparable to the untreated control (Figure 5A), whereas PI fluorescence remained strongly reduced throughout the experiment (Figure 5B). This behavior aligns with prior reports summarized by Okshevsky et al. [37], which indicate that DNase I can disrupt or disperse established *E. coli* biofilms but is generally ineffective at preventing biofilm formation altogether, suggesting that eDNA is particularly critical during early attachment and matrix establishment rather than during later biomass accumulation.

Morphological inspection further revealed striking differences in streamer architecture (Figure S19). In the untreated culture, early

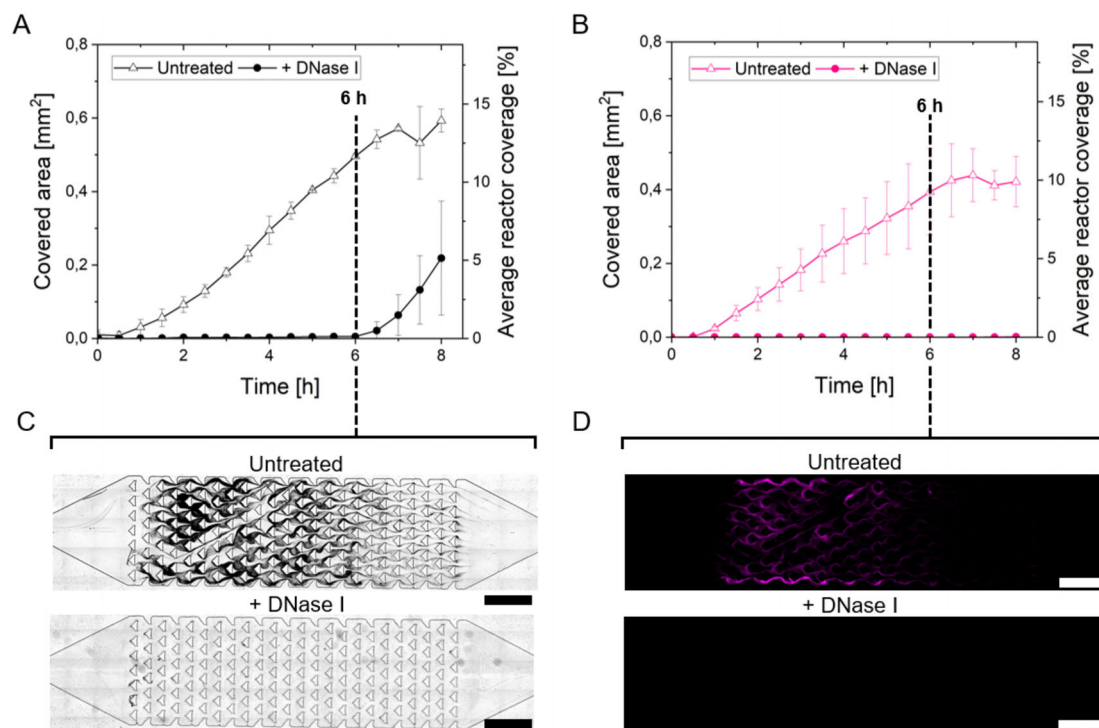


FIGURE 5 | Effect of DNase I on the deposition behavior of *E. coli* eGFP cells in a microfluidic R2_40 reactor. Cell suspensions containing PI ($c_{PI} = 4 \mu\text{g mL}^{-1}$) were injected at $16 \mu\text{L min}^{-1}$ for 8 h, either untreated (control) or supplemented with DNase I (2 mg mL^{-1}). Quantification of reactor coverage by (A) biomass and (B) PI ($\lambda_{\text{exc}} \sim 535 \text{ nm}$, $\lambda_{\text{em}} \sim 617 \text{ nm}$) fluorescence over time shows a pronounced delay in deposition for the DNase-treated sample, with a rapid increase in biomass occurring only after approximately 6 h. While biomass coverage eventually approaches that of the control, PI-associated signals remain markedly reduced at identical imaging settings, indicating lower apparent DNA availability. (C) Representative bright-field and PI fluorescence images (D) after 6 h of injection for the untreated (upper row) and DNase-treated (lower row) samples. Streamer formation is evident in the control, whereas DNase I treatment strongly delays early deposition and results in substantially weaker PI signals under the same acquisition conditions. Flow direction from left to right. Scale bar: 1 mm.

streamers (1.5 h) exhibited fine, filamentous structures composed of individual cell chains. By 7.5 h, the untreated streamers had elongated but remained delicate and visibly structured. In contrast, streamers forming in the DNase-treated culture after the 6 h delay were markedly more compact, lacking the filamentous cell-chain morphology characteristic of untreated samples. Their surfaces appeared rounded and smooth, and PI fluorescence was barely detectable even after 7.5 h, indicating severely reduced eDNA availability within the matrix.

These observations demonstrate that extracellular DNA is required for the rapid formation of early streamer structures and contributes to the mechanical stability of their filamentous architecture. At the same time, the absence of early PI signals in DNase-treated samples indicates that streamer formation can, under certain conditions, proceed with strongly reduced eDNA content, possibly relying on enhanced autoaggregation [38] or alternative extracellular polymeric substances such as exopolysaccharides, proteins or curli fibres [30, 39]. This finding aligns with the broader picture summarized by Okshevsky et al., who reported pronounced species- and stage-dependent effects of DNase I on biofilms and noted that, for *E. coli*, DNase I is generally effective in disrupting established biofilms but not in preventing initial biofilm formation [37]. The delayed onset of deposition observed here may therefore reflect regulatory adaptation processes, for example through altered expression of surface-associated components under modified growth and

attachment conditions [40]. Quorum-sensing pathways may further contribute to coordinated matrix restructuring in response to reduced eDNA availability [41].

Finally, DNase I may already affect cells in suspension prior to deposition, promoting agglomeration and thereby enabling larger cell clusters to become trapped between pillars. A gradual reduction of DNase activity over time, for instance through proteolytic degradation by the cells, may further contribute to the eventual formation of compact streamers. Despite these potential confounding influences, the present results help to experimentally refine an open question in the literature by showing that, under continuous-flow conditions, eDNA is particularly critical for the early stages of streamer nucleation, while later matrix assembly can partially compensate for its depletion. This underscores the central, although not exclusive, role of eDNA in early streamer formation and demonstrates that *E. coli* can adopt alternative matrix-building strategies when eDNA availability is reduced.

2.4 | Competitive Deposition and Spatial Persistence of Biofilms Under Continuous Flow

Following the identification of stable, pillar-associated biofilm structures formed under continuous flow, we next asked how robust these deposits are when exposed to newly arriving cells.

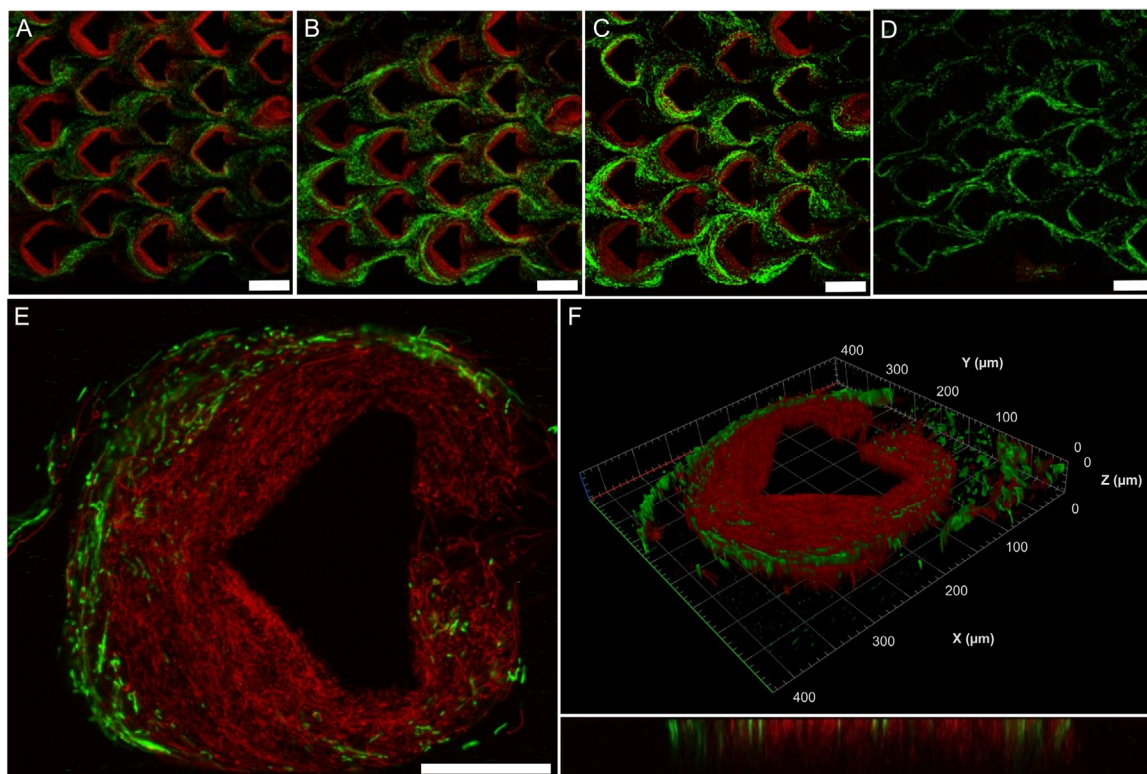


FIGURE 6 | Dynamic displacement of pre-deposited *E. coli* cells in a microfluidic reactor. (A–D) CLSM images showing the temporal progression of a displacement experiment. An *E. coli* mRFP ($\lambda_{\text{exc}} \sim 558$ nm, $\lambda_{\text{em}} \sim 583$ nm) suspension was injected for 15.5 h at $16 \mu\text{L min}^{-1}$ to establish a red-labeled biomass layer. Subsequently, an *E. coli* eGFP ($\lambda_{\text{exc}} \sim 488$ nm, $\lambda_{\text{em}} \sim 507$ nm) suspension was perfused under identical conditions, and displacement of the pre-deposited biomass was monitored over 8 h. Images correspond to (A) 2 h, (B) 3 h, (C) 4 h and (D) 8 h after initiation of displacement. (E) High-resolution CLSM image of a representative deposition event after 5 h 20 min, showing green cells attaching to a pre-existing red biomass layer at a pillar surface. (F) 3D CLSM stack of the same region, illustrating the spatial arrangement of red and green cells within the deposited biomass after 4 h 30 min. Flow direction from left to right. Reactor: R2_40. Scale bars: A–D = 200 μm , E = 100 μm .

To address this, we developed a displacement experiment that allowed us to visualize competitive deposition dynamics in real time and to assess whether newly introduced cells can displace or overgrow pre-existing biomass within the microfluidic reactor (Figure 6). This approach provides insight into the spatial persistence of established biofilms and identifies reactor regions that support long-term biomass retention.

For this purpose, *E. coli* strains expressing either eGFP or mRFP were used as fluorescently distinct populations. First, an *E. coli* mRFP suspension adjusted to $\text{OD}_{600} = 0.05$ was perfused through an R2_40 reactor for 15.5 h at $16 \mu\text{L min}^{-1}$ to generate a mature layer of red-labeled biomass. Subsequently, the flow was switched via a three-way valve (Figure S10C) to an *E. coli* eGFP suspension adjusted to $\text{OD}_{600} = 0.05$, which was injected under identical conditions. The displacement process was monitored over 8 h using CLSM, complemented by high-resolution imaging of individual deposition events at pillar surfaces and a 3D Z-stack of the biomass (Figure 6E,F).

Initially, the reactor was densely populated with red-labeled cells, which formed continuous deposits and occasional streamers. Over the course of 8 h, these red cells were progressively displaced by the incoming green population (Figure 6A–D). Although the majority of red biomass was removed or overgrown, residual red cells remained detectable even after 4 h of displacement

(Figure 6C), particularly at the pillar tips. In contrast, the near absence of red streamers at later time points suggests that, in this setup, streamers occur only transiently compared with pillar-associated biomass.

Higher magnification imaging of individual deposition events showed that green cells accumulated preferentially on top of the existing red biomass layer at pillar surfaces, while only a few green cells penetrated into the underlying red matrix (Figure 6E). A 3D Z-stack of the same region confirmed that deposition of eGFP-expressing cells initiates near the bottom substrate (Figure 6F), suggesting that new biomass preferentially attaches either at exposed surfaces of the existing deposit or directly at the pillar–substrate interface.

Taken together, these observations demonstrate that biofilms formed in the microfluidic reactor are structurally stable and resilient to displacement, whereas streamer-based biomass is more easily removed. The preferential persistence of pillar-associated biomass supports the idea that stable biofilm formation in this system is localized primarily at pillar tips, where hydrodynamic forces and surface geometry favor long-term attachment. Beyond demonstrating robustness, the displacement experiments highlight the potential of this platform for studying competitive or cooperative colonization dynamics, sequential biofilm layering or multispecies interactions. In future

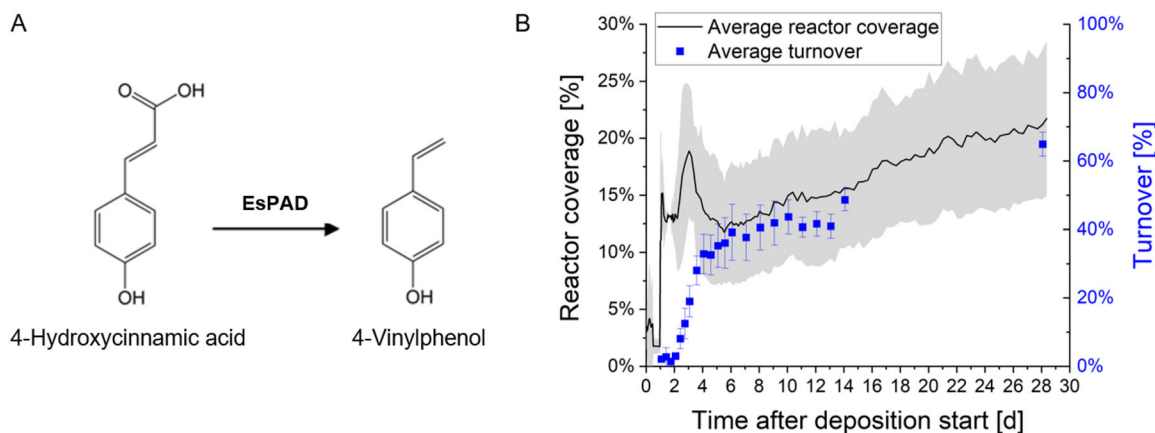


FIGURE 7 | Continuous whole-cell biocatalysis in the scaled reactor R4_160. (A) Reaction scheme for the EsPAD-catalyzed decarboxylation of 4-hydroxycinnamic acid (HCA) to 4-vinylphenol. (B) Time course of biomass coverage (black) and EsPAD turnover (blue) during continuous operation. *E. coli* cells expressing EsPAD were deposited for 20 h at $128 \mu\text{L min}^{-1}$, after which a medium containing 5 mM HCA was perfused at $35 \mu\text{L min}^{-1}$ to enable continuous biocatalysis for 28 days. Turnover was quantified by HPLC analysis of effluent samples. Data represent triplicate experiments. Note that a transient increase in apparent reactor coverage after switching from the turbid deposition suspension to the transparent substrate solution is attributable to optical contrast effects as well as biofilm regrowth and reorganization under continuous flow.

applications, this setup could be combined with micro-sampling approaches [42, 43] and transcriptomic profiling to investigate spatial gene expression patterns within stratified biofilms.

2.5 | Continuous Whole-Cell Biocatalysis Enabled by a Scaled Flow-Deposition Reactor

To translate the flow-induced deposition concept from mechanistic biofilm studies to functional application, we next evaluated whether the scaled reactor geometries developed in this work can sustain continuous whole-cell biocatalysis under long-term operation. This investigation builds directly on the preceding results, which demonstrated that hydrodynamic scaling preserves deposition-relevant flow features and enables the formation of stable, pillar-associated biofilms in enlarged reactor geometries. As a performance benchmark, we selected the EsPAD-catalyzed decarboxylation of 4-hydroxycinnamic acid to 4-vinylphenol, a well-established model reaction for assessing whole-cell catalytic performance [24, 43]. The experiment thus served both as a functional validation of the upscaled reactor architecture and as a proof-of-concept for continuous whole-cell biocatalysis under preparative flow conditions.

Because the larger reactor geometry (R4_160) precluded high-resolution fluorescence imaging and generated substantially higher effluent volumes, a dedicated imaging and sampling setup was established (Figure S20). Three *E. coli* suspensions expressing EsPAD were deposited in parallel for 20 h, after which the cell suspension was replaced by medium supplemented with 4-hydroxycinnamic acid to initiate continuous biocatalytic conversion. The reactors were operated using a peristaltic pump to achieve the required high flow rates, while continuous stirring ensured homogeneous substrate supply. Biomass accumulation at the reactor outlets was monitored by transmitted-light imaging using bottom illumination and a mounted digital camera. Defined effluent volumes were collected at regular intervals using an automated sampling system, enzymatic activity was quenched

immediately, and product formation was quantified by HPLC (Figure S21). Reactor coverage was extracted from time-resolved images and expressed as the average fraction of biomass-covered area (Figure S20C).

Following the deposition phase, the reactors initially exhibited low biomass coverage and only minimal EsPAD activity (Figure 7B). Over the subsequent cultivation period, biofilm-associated biomass increased and continuous product formation was established, reaching steady conversion levels after several days of operation. The simultaneous increase in biofilm coverage and product formation demonstrates that biofilm growth and catalytic turnover proceed in parallel under the applied reaction conditions. Notably, the biofilms remained stable throughout the entire catalytic operation, which extended to 28 days without detectable detachment or loss of activity. Together, these observations show that continuous catalytic turnover is fully compatible with sustained biofilm development in the flow-deposition reactor. Under these conditions, the maximum product conversion approached approximately 64%, corresponding to a space-time yield of $\sim 1800 \text{ g L}^{-1} \text{ d}^{-1}$. While this value is lower than the space-time yield previously reported for the original small-scale reactor [24], it demonstrates that the upscaled reactor R4_160 supports sustained whole-cell catalysis under continuous-flow conditions.

Compared with batch operation, continuous-flow processing maintains a quasi-steady reaction environment with constant substrate supply and continuous product removal, thereby supporting stable catalytic performance of the immobilized biofilm. Furthermore, in contrast to immobilized enzyme systems, intact biofilm-associated cells retain the capacity for cofactor regeneration and enable a self-maintaining catalytic matrix. Continuous biomass turnover within the biofilm, resulting from the balance between growth and shear-induced detachment, allows the catalytic system to effectively self-repair during long-term operation. Such reactor concepts are increasingly discussed in the context of microscale whole-cell bioprocessing and so-called flow fermentation, where continuous perfusion and precise

environmental control enable sustained microbial production processes [18]. Importantly, continuous convective transport replenishes substrates and removes products at the biofilm interface, thereby helping to mitigate potential mass transfer limitations associated with biofilm-based catalysts. Consistent with this interpretation, time-resolved PI staining experiments (Figure S11) indicate that small molecules can penetrate the biofilm matrix under flow conditions.

Notably, the deposited biofilms remained catalytically active throughout the entire 28-day operation, with no indication of catastrophic detachment or loss of function. Importantly, no clogging of the reactor channels was observed during this period of continuous operation, suggesting that the dynamic balance between biomass growth and shear-induced detachment prevents excessive accumulation and allows detached biomass fragments to be flushed out of the reactor. This behavior is consistent with previous studies highlighting the role of shear-driven dynamics in regulating biofilm accumulation and restructuring under flow conditions [44]. Given the scarcity of reports on long-term productive biofilms operated under continuous flow, this level of operational stability highlights the robustness of the flow-induced deposition principle when transferred to larger reactor geometries. While absolute space–time yields decrease upon upscaling, hydrodynamic scaling preserves the functional integrity of the biofilm, enabling prolonged and reproducible whole-cell catalysis under conditions relevant for preparative flow operation. Together, these results establish scaled flow-deposition reactors as a promising platform for long-duration continuous biocatalysis, bridging microscale biofilm engineering with stable, application-oriented reactor performance.

3 | Conclusions

This work establishes flow-structured microfluidic reactors as a materials-inspired platform for the controlled formation and functional use of living biofilms. We show that small geometric features and hydrodynamically defined shear fields act as design elements that direct early cell attachment, streamer nucleation and the emergence of stratified, mechanically resilient biofilm architectures under continuous flow. These architectures persist over extended operation times, demonstrating that biofilms formed by flow-induced deposition behave as stable, self-organized living materials rather than transient accumulations.

Systematic scaling of the reactor geometry, supported by CFD analysis, reveals that preserving deposition-relevant microscale flow features is sufficient to maintain biofilm formation while improving manufacturability and operational robustness. Matrix-sensitive imaging further shows that extracellular DNA-rich structures are spatially organized by these microscale environments, highlighting how small hydrodynamic differences translate into distinct structural properties within the biofilm. By coupling these structural insights with continuous whole-cell catalysis in an upscaled reactor, we demonstrate that microscale architectural control can be translated into macroscopic function.

Together, our results define a generalizable reactor concept in which modular design, hydrodynamic patterning through reactor geometries that generate spatially structured flow fields guiding

localized biomass deposition, and long-term stability converge, providing a versatile framework for studying and engineering biofilms as functional soft materials in both fundamental and applied settings. Related flow-driven assembly processes have been reported in other biological and biomaterial systems, including the shear-induced organization of von Willebrand factor in microfluidic channels [45] and the formation of dynamic DNA-based biomaterials via shear-induced deposition during rolling-circle amplification in micropillar reactors [46]. Emerging data-driven approaches further highlight the potential of hydrodynamic information for guiding rational microreactor design [47].

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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 from the corresponding author upon reasonable request.

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Supporting Information

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

Supporting File: smll73391-sup-0001-SuppMat.pdf.