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Modular Assembly of a Biocatalytic Material for Stereoselective C–Si Bond Formation

Marc F. Munker  | Annika J. Weber  | Madleen Richter  | Jennifer Kühne  | Carmen M. Dominguez  | Christof M. Niemeyer  | Kersten S. Rabe 

Institute For Biological Interfaces 1 (IBG-1), Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany

Correspondence: Kersten S. Rabe (kersten.rabe@kit.edu)

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ABSTRACT

Biocatalysis offers sustainable routes to complex molecules, yet translating enzymatic activity into robust, reusable materials remains a major challenge. Here, we report the modular assembly of a self-supporting biocatalytic material for stereoselective C–Si bond formation using an engineered *Rhodothermus marinus* cytochrome c (CC). The material was constructed by site-specific covalent coupling of CC fused with two SpyCatcher domains (SC-CC-SC) with streptavidin variants bearing SpyTag domains (STV-ST), yielding a red, porous enzyme foam with defined hexagonal architecture. The modular STV-mediated crosslinking strategy enables the formation of carrier-free materials with high enzyme density and minimal diffusion barriers. The CC foams retained structural integrity and catalytic activity after drying, catalyzing the formation of a chiral organosilicon compound with high enantioselectivity under mild conditions. The enzyme remains active at the extensive gas–liquid interfaces within the foam, effectively combining high catalyst loading with excellent mass transport. The resulting material retains high stereoselectivity (>95% ee), exhibits catalytic performance comparable to previously reported carrier-based immobilization approaches, and maintains catalytic activity over five consecutive reuse cycles. This immobilization concept establishes a generalizable platform for fabricating biocatalytic materials from monomeric enzymes and expands the scope of enzymatic C–Si bond formation toward scalable, sustainable synthesis.

1 | Introduction

Biocatalysis is increasingly recognized as a central technology for the sustainable production of chemicals and materials, offering selective and energy-efficient transformations under mild conditions [1–3]. The combination of enzyme engineering and reaction-cascade design has greatly expanded the synthetic repertoire of biocatalysis, enabling transformations that were once considered beyond the reach of biology [4–9]. Through directed evolution, enzymes have been adapted to perform new-to-nature

reactions, including C–C, C–N, and more recently, C–Si bond formations processes traditionally dominated by transition-metal catalysis [10–13].

Heme proteins, with their tunable active-site architectures and versatile iron cofactors, have proven particularly powerful in this context [14–20]. A notable example is the engineered *Rhodothermus marinus* cytochrome c (V75T M100D M103E, abbreviated here as CC), which catalyzes the enantioselective formation of silicon–carbon bonds [21, 22]. This breakthrough demonstrates

Marc F. Munker and Annika J. Weber contributed equally to this work.

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that biocatalysis can access organosilicon chemistry, unlocking sustainable routes to valuable chiral organosilicon compounds relevant to pharmaceuticals and materials science [23–25].

To translate such laboratory achievements into practical, scalable processes, the stability, reusability, and integration of biocatalysts into continuous operation must be addressed [26–28]. Enzyme immobilization provides a powerful strategy to meet these challenges by enhancing robustness and enabling catalyst recovery. However, conventional carrier-based approaches often suffer from low enzyme loading and mass transfer limitations, which can severely affect productivity in flow systems [5, 29, 30].

Emerging carrier-free biocatalytic materials overcome the limitations of conventional carrier systems by forming self-supporting, densely packed enzyme networks with intrinsically high volumetric activity. These assemblies combine the benefits of immobilization with minimal diffusion barriers and are particularly attractive for continuous-flow operation. Self-immobilized enzyme matrices generated through cross-linking provide very high catalyst loadings in the reactor without requiring inert carriers [31–35], while the surrounding protein environment helps to maintain a natural hydration shell that supports structural integrity and catalytic performance [36]. However, conventional cross-linking methods typically rely on chemical reagents that can compromise enzyme activity and limit applicability to sensitive or engineered biocatalysts.

To overcome these constraints, genetically encoded coupling systems such as SpyCatcher/SpyTag (SC/ST) [37–50] and SnoopCatcher/SnoopTag (SnC/SnT) [49, 51–55] offer a gentle and site-specific route to self-assembly. In these systems, the complementary domains spontaneously form an intermolecular isopeptide bond upon recognition, yielding stable covalent linkages under physiological conditions. The SC/ST system, in particular, has been successfully applied to diverse enzyme classes [56–62], enabling the formation of robust and catalytically active biocatalytic networks without chemical crosslinkers.

Building on these concepts, we previously equipped the C–Si bond-forming CC with an N-terminal ST (ST-CC) to enable directed immobilization on particulate carriers [63, 64]. While these systems demonstrated site-specific and robust enzyme attachment, they were restricted to carrier-based architectures. To advance toward fully self-supporting materials suitable for continuous flow biocatalysis, we now translate this concept into carrier-free, protein-only assemblies. Here, we report the development of all-enzyme hydrogel (AEH) and all-protein hydrogel (APH) materials based on the C–Si bond-forming CC. Two complementary strategies were pursued: (i) AEH structures composed exclusively of CC variants bearing multiple coupling tags to achieve autonomous crosslinking, and (ii) APH materials combining monomeric CC fusions with multimeric scaffold proteins carrying coupling domains on each subunit. The latter design provides a well-defined protein framework that is catalytically inert and crosslinks enzymatic building blocks while maintaining full accessibility of their active sites, in contrast to small molecule crosslinkers that often hinder access.

This study represents the first application of a new-to-nature C–Si bond-forming enzyme system for the creation of self-

assembling, carrier-free biocatalytic foams, merging high enzyme density and efficient mass transport within a purely proteinaceous matrix [61, 62]. These results establish a robust platform for transforming monomeric, engineered biocatalysts into functional materials and open new avenues for more sustainable catalysis in continuous processes.

2 | Results and Discussion

2.1 | Design of Cytochrome c Building Blocks for All-Enzyme Biocatalytic Materials

To establish an all-enzyme hydrogel (AEH) composed solely of the C–Si bond-forming cytochrome c from *R. marinus* (CC), we initially employed our previously developed N-terminal ST fusion (ST-CC) [63, 64]. However, earlier work revealed a substantial reduction in expression yield for this construct, as two protein species were produced, one of which contained a nonfunctional ST [63]. Although cultivation in TB medium instead of LB medium markedly improved total expression levels (Figure S1), the partial cleavage of the N-terminal tag was not mitigated by this change.

To circumvent this limitation, we replaced the SpyCatcher/SpyTag (SC/ST) system with the SnoopCatcher/SnoopTag (SnC/SnT) system [51], which features a distinct amino acid sequence and is therefore less prone to proteolytic cleavage by periplasmic enzymes. Using this alternative strategy, we designed a series of SnT- and SnC-functionalized CC variants to enable self-assembly into hydrogel structures. Specifically, three different SnT-CC constructs (variants I–III) and one double-tagged SnC-CC-SnC construct (variant IV) were generated by recombinant expression, following previously established procedures (Figure 1a) [63, 64].

The SnT-functionalized variants of CC differ in the number and position of the SnT domains (Figure 1a, bright green circles) relative to CC (Figure 1a, orange enzyme structure) (for a detailed view of the sequence see Figure S2a). Variant I carries a single SnT at each terminus (Figure 1a, black), variant II features three SnT domains, one at each terminus and an additional one inserted within the CC sequence, thereby interrupting the native protein chain (Figure 1a, gray), and variant III contains two consecutive SnT domains directly adjacent to the C-terminus (Figure 1a, pink). To assess whether these modifications might sterically affect protein folding or tag accessibility, structural models of all SnT variants were generated using AlphaFold2 (Figure S2b), indicated that these modifications should not sterically affect protein folding or tag accessibility [65]. To further evaluate the dynamic behavior of the fusion proteins in solution, molecular dynamics (MD) simulations were performed for all variants. As expected, the simulations revealed substantial conformational flexibility of the fusion constructs, reflected by the RMSD trajectories, with the largest structural fluctuations observed for SnT-CC(SnT)-SnT (II) (Figure S3). Root-mean-square fluctuation (RMSF) analysis further demonstrated that the regions containing the inserted SnT domains exhibit enhanced flexibility, whereas the CC core remained comparatively rigid throughout the simulations (Figure S4). These results indicate that insertion of the coupling domains does not substantially affect the structural integrity of the

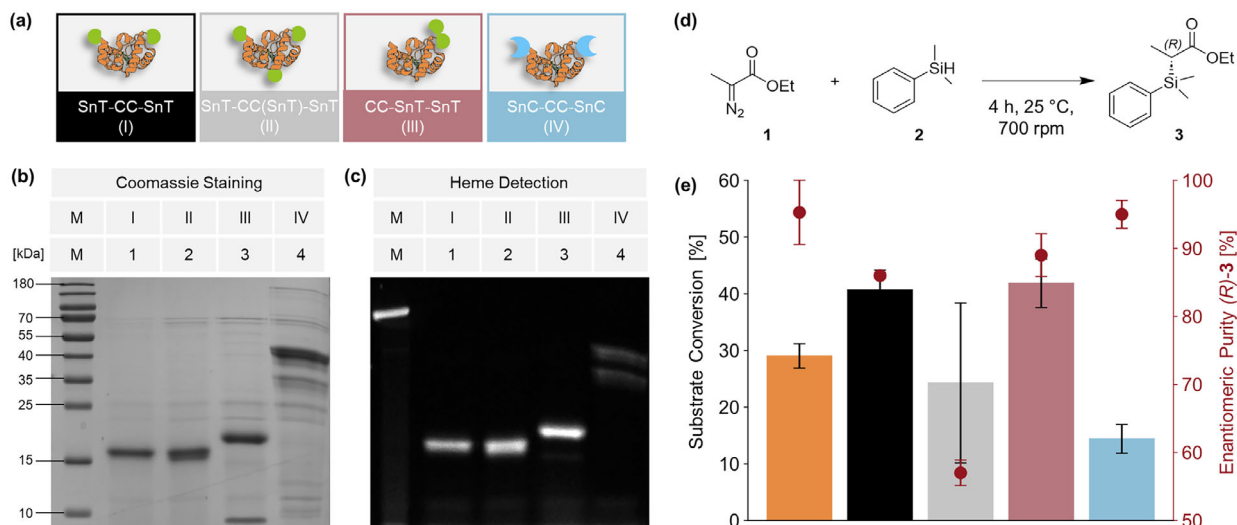


FIGURE 1 | Variants of genetically fused *Rhodothermus marinus* cytochrome c (CC) with different numbers and positions of Snoop-Catcher/SnoopTag (SnC/SnT) domains. (a) Schematic representation of the SnC/SnT-functionalized CC variants produced: SnT-CC-SnT (I, black), SnT-CC(SnT)-SnT (II, gray), CC-SnT-SnT (III, pink), and SnC-CC-SnC (IV, blue). For details, see Figure S2. (b) SDS-PAGE analysis with subsequent Coomassie staining of purified CC variants I–IV (lanes 1–4). All proteins were expressed in TB medium and purified via affinity chromatography; molecular weight marker (M) in kDa. (c) Heme-specific detection of the same gel shown in (b). (d) Catalytic activity of the SnC/SnT-functionalized CC variants (I–IV) compared to the unmodified CC (orange) in the model reaction of ethyl 2-diazopropanoate (**1**) and phenyl dimethylsilane (**2**) to form the organosilicon product (**3**). (e) Substrate conversion (bars) and enantiomeric excess of **3** (circles) in batch reactions containing 1 μ M enzyme, 10 mM NaDT, 10 mM **1** and **2** for 4 h under anaerobic conditions at room temperature; conversion determined by GC and enantiomeric purity by chiral HPLC as previously described. Error bars represent the standard deviation from duplicates.

catalytic CC domain while providing the flexibility required for subsequent self-assembly. In addition, a construct bearing a SnC domain at both termini (SnC-CC-SnC, variant IV) was designed to enable potential coupling with ST-modified proteins (Figure 1a, blue).

SDS-PAGE and heme-specific fluorescence analyses confirmed successful expression and purification of the SnT variants I–III, each appearing as single bands after Coomassie staining (Figure 1b) and heme detection (Figure 1c) [66]. In contrast, SnC-CC-SnC (IV) displayed a double band with reduced fluorescence intensity, indicating lower expression yield and partial instability. This variant also showed a tendency to precipitate at higher enzyme concentrations, suggesting limited solubility. Nevertheless, all SnC/SnT variants were obtained in sufficient quantities to investigate their enzymatic activity. Their activity in silicon-carbon bond formation was evaluated using the established model reaction between ethyl 2-diazopropanoate (**1**) and phenyl dimethylsilane (**2**) [21], yielding the chiral organosilicon product (**3**), as quantified by GC and analyzed for enantiomeric purity by chiral HPLC (Figure 1d) [63]. Variants I and III catalyzed the reaction with satisfactory substrate conversion and high enantioselectivity (Figure 1e, black and pink), whereas variant II (gray) displayed poor stereocontrol, which might be due to the rather high flexibility observed in the MD simulation resulting in an overall reduced performance, and was thus not further considered. SnC-CC-SnC (IV, blue) retained high enantioselectivity but showed markedly reduced turnover compared to the unmodified CC (orange).

Since the formation of all-enzyme hydrogels requires large amounts of stable, soluble, and active enzyme, the SnC-CC-SnC construct was deemed unsuitable for AEH preparation. Con-

sequently, subsequent work focused on developing alternative strategies for self-assembling biocatalytic materials based on the more stable SnT-tagged CC variants.

2.2 | Development of a Multimeric Protein Scaffold for All-Protein Biocatalytic Hydrogels

Recognizing the limitations of the direct AEH approach, we next pursued a conceptually distinct strategy to construct all-protein hydrogels (APH) based on a multimeric scaffold protein capable of crosslinking enzymatic building blocks. For this purpose, the well-characterized and exceptionally stable tetrameric protein streptavidin (STV) was chosen as a biocompatible crosslinker [67–73]. Importantly, STV is catalytically inert toward our silicon-carbon bond-forming model reaction (Figure 2a) [74–76], making it ideally suited as a structural element for biocatalytic materials.

Beyond its stability and availability, STV has been successfully employed as a modular component in protein network formation through fusions with the SC/ST system [38, 50, 77], providing an attractive precedent for our design. Here, we extended this concept by engineering SnC- and SnT-fused streptavidin variants (STV-SnC and STV-SnT) to enable self-assembly with the corresponding SnC/SnT-tagged CC variants (Figure 1). This approach introduces a versatile protein-only crosslinking strategy for creating defined, hybrid biocatalytic networks (Figure 2a, bright blue and bright green). In parallel, we devised an alternative configuration employing the complementary SC/ST system. In this design, the periplasmically expressed ST-CC construct is replaced by a cytosolic STV-ST fusion (dark green), which can assemble with the SC-CC-SC enzyme variant (dark blue) to form a protein-embedded hydrogel (APH) without relying on periplasmic expression of tagged enzymes.

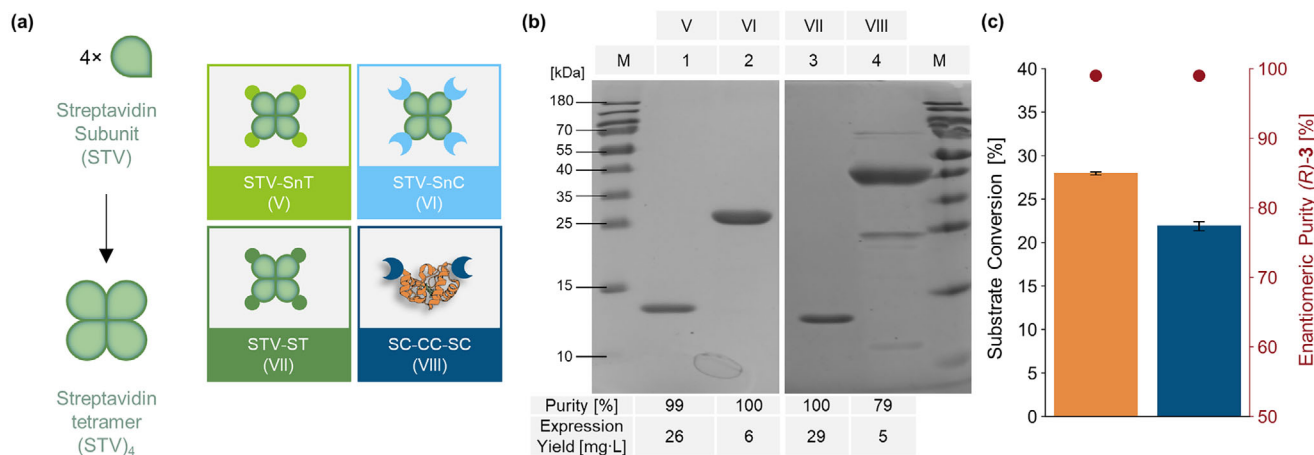


FIGURE 2 | Protein fusions for the formation of hydrogels composed of CC and streptavidin (STV) used in this study. (a) Concept of using the tetrameric STV as a crosslinking protein for polymerizing monomeric CC. STV consists of four identical subunits and C-terminal fusions were generated with a SnoopTag (STV-SnT, bright green, V), a SnoopCatcher (STV-SnC, bright blue, VI), or a SpyTag (STV-ST, dark green, VII). To enable polymerization via the SpyCatcher/SpyTag system, a CC variant carrying SpyCatcher domains at both termini (SC-CC-SC, dark blue, VIII) was constructed. (b) SDS-PAGE analysis with subsequent Coomassie staining of purified STV and CC fusion proteins V–VIII (lanes 1–4); molecular weight marker (M) in kDa. (c) Substrate conversion (bars) and enantiomeric excess of product 3 (circles) obtained in batch reactions employing 5 μ M unmodified CC (orange) or SC-CC-SC (dark blue) with 10 mM NaDT, 10 mM **1** and **2** under anaerobic conditions for 4 h at room temperature. Error bars represent the standard deviation from duplicates.

The STV variants were generated by inserting the coupling domains at the C-terminus of the streptavidin sequence based on a reported plasmid template [78]. Following heterologous expression in *Escherichia coli*, the proteins were refolded from inclusion bodies and purified to high homogeneity by iminobiotin affinity chromatography (Figure 2b, lanes 1–3). Likewise, the SC-CC-SC enzyme fusion was successfully expressed and purified, yielding a single band on SDS-PAGE (Figure 2b, lane 4). Functional assays confirmed that SC-CC-SC retained catalytic activity in the model reaction between ethyl 2-diazopropanoate (**1**) and phenyl dimethylsilane (**2**) to afford organosilicon **3**, comparable to that of unmodified CC (Figure 2c).

2.3 | Formation and Characterization of CC–Streptavidin All-Protein Biocatalytic Materials

Based on the characterization of the individual components, three complementary pairs were selected for all-protein hydrogel (APH) formation: SnT-CC-SnT with STV-SnC (I + VI, Figure 3a, black), SnT-CC(SnT)-SnT with STV-SnC (II + VI, gray), and SC-CC-SC with STV-ST (VIII + VII, blue). Upon mixing the complementary partners at equimolar ratios of the coupling domains, covalent crosslinking via spontaneous isopeptide bond formation was expected to yield APH assemblies (Figure 3b).

Particle formation was monitored by dynamic light scattering (DLS) to follow the increase in hydrodynamic diameter over time after mixing the two coupling partners. This approach has previously proven valuable for characterizing all-enzyme hydrogels [56, 58–60], which typically yield nanogel particles with hydrodynamic diameter around 50 nm after 2 h using 1 mM protein solutions. Under analogous conditions, the SnT-CC-SnT/STV-SnC (black) and SnT-CC(SnT)-SnT/STV-SnC (gray) mixtures formed stable nanoscale assemblies with hydrodynamic diam-

eters of approximately 30 and 50 nm, respectively (Figure 3c). The formation of these well-defined particles confirms successful covalent coupling between the complementary domains, while the observed size variation likely reflects differences in the effective valency and spatial arrangement of the SnT domains within the CC variants. In comparison, mixing SC-CC-SC with STV-ST (blue) resulted in continuous particle growth, reaching a hydrodynamic diameter of approximately 75 nm after 2 h (Figure 3c), consistent with highly efficient crosslinking and the formation of extended protein networks.

To confirm that the observed particle formation originated from specific covalent crosslinking rather than nonspecific aggregation, the SC-CC-SC/STV-ST mixtures were analyzed by SDS-PAGE after DLS measurements (Figure 3d,e). A distinct coupling product with an apparent molecular weight of $\approx$ 55 kDa was detected, consistent with the expected conjugate of SC-CC-SC covalently bound to two STV-ST molecules.

The crosslinking functionality of STV-ST was further validated using a previously established tetrameric SC-ADH [56], which produced the expected 50 nm hydrogel particles (Figure S5). Moreover, the preserved biotin-binding capability of STV-ST was confirmed by interaction with biotinylated DNA, detected via PAGE (Figure S6). Collectively, these results establish the SC-CC-SC/STV-ST pair as a robust foundation for the formation of functional biocatalytic materials.

2.4 | Development of CC–Based Biocatalytic Enzyme Foams

We have recently established biocatalytic enzyme foams [61, 62], which show superior catalytic performance when compared to monolithic hydrogels, resulting from their high enzyme loading

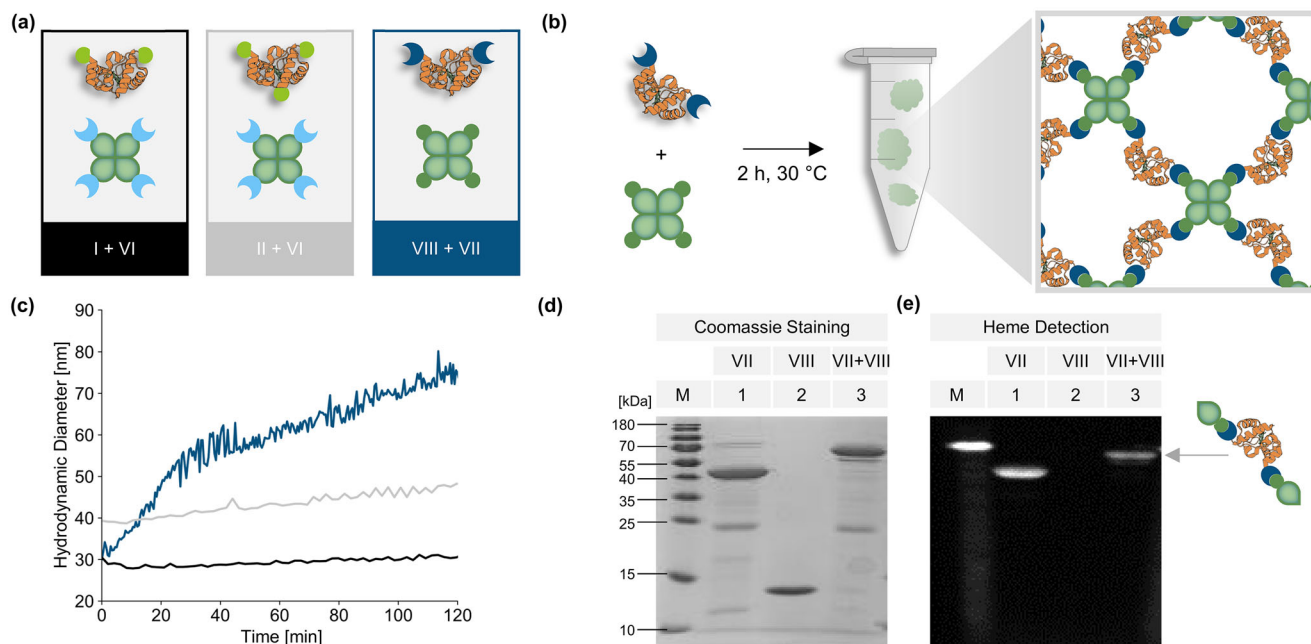


FIGURE 3 | Formation of CC all-protein hydrogel (APH) particles. (a) Protein combinations used for APH formation: SnT-CC-SnT/STV-SnC (I/VI, black), SnT-CC(SnT)-SnT/STV-SnC (II/VI, gray), and SC-CC-SC/STV-ST (VIII/VII, blue). (b) Schematic illustration of CC hydrogel formation using SC-CC-SC and tetrameric streptavidin (STV-ST)₄, in which specific covalent coupling between SC and ST domains drives polymer network formation. (c) Time-dependent increase in the hydrodynamic diameter of CC hydrogel particles determined by dynamic light scattering (DLS). Protein concentrations were adjusted to the stoichiometry of the coupling domains: 666 μ M SnT-CC-SnT and 333 μ M STV-SnC (black), 570 μ M SnT-CC(SnT)-SnT and 430 μ M STV-SnC (gray), and 333 μ M SC-CC-SC and 666 μ M STV-ST (blue). Measurements were performed at 30°C over 2 h. (d) SDS-PAGE with subsequent Coomassie staining of purified SC-CC-SC and STV-ST before coupling and after a two-hour incubation, confirming formation of CC-APH conjugates; molecular weight marker (M) in kDa. (e) Heme-specific detection of the same gel as in (d), verifying the covalent attachment of the heme-containing SC-CC-CC with two STV-ST.

and large surface-to-volume ratios. Therefore, we next transferred the cytochrome c-streptavidin system directly into a foamed material format. Such biocatalytic foams efficiently overcome diffusion and mass transport limitations that typically restrict the activity of dense hydrogel matrices [61, 62]. Following the optimized coupling conditions for APH formation, a mixture containing 333 μ M SC-CC-SC and 666 μ M STV-ST (1 mM total enzyme concentration in PB) was subjected to foaming in a custom flow-focusing setup driven by nitrogen influx, as previously described [61, 62]. The resulting wet foam exhibited a distinct orange coloration, serving as a clear visual indicator of the successful incorporation of the heme-containing CC into the material matrix (Figure 4a). The foam consisted of monodisperse bubbles with a homogeneous distribution immediately after formation, demonstrating that the protein system remained structurally intact during the foaming process. However, after drying for 2 days at 30°C (Figure 4b), the material retained a predominantly spherical bubble morphology rather than developing the characteristic hexagonal pore structure previously observed in other enzyme foams [61, 62]. Progressive coalescence of adjacent bubbles led to the formation of larger cavities, indicating that while covalent crosslinking occurred effectively, the structural stability of the foam matrix was still limited under the current conditions.

To further improve the stability of the cytochrome c foams, we next aimed to introduce surface-active components that could strengthen the gas-liquid interfaces of the material while maintaining the functionality of the SC/ST-mediated protein network.

Foam stability is a critical parameter for continuous biocatalytic applications, as it determines both the operational lifetime and the mechanical integrity of the material. In a first approach, we sought to modify the SC domain to allow chemical attachment of amphiphilic molecules, thereby endowing the system with interfacial activity. For this purpose, four SC mutants carrying a single reactive cysteine residue were generated, enabling selective conjugation with amphiphilic molecules through maleimide chemistry (Figures S7 and S8). Subsequent coupling attempts using a hydrazide linker to connect the cysteine thiol to the aldehyde functionality of saponin [79], a natural surfactant [80, 81], yielded only small amounts of the desired conjugate and thus proved unsuitable for foam generation (Figure S9).

As a more promising, cost-efficient, and modular alternative to chemical surfactant conjugation, we turned to protein-based surfactants that naturally stabilize foams. The frog nest protein ranaspumin-2 (Rsn2), known for its strong interfacial activity and ability to maintain stable foams in aqueous environments [82, 83], was selected as a bioinspired and readily scalable functional module. To integrate this surface-active property into our modular SC/ST coupling framework, a genetic fusion of SC and Rsn2 (SC-Rsn2) was recombinantly produced. The purified fusion protein displayed high expression levels, high purity, and retained full SC functionality, as evidenced by the formation of a specific coupling product with ST-GFP [84] (Figure S10). The SC-Rsn2 construct was subsequently implemented in a previously established model cascade system [56, 61], consisting of alcohol

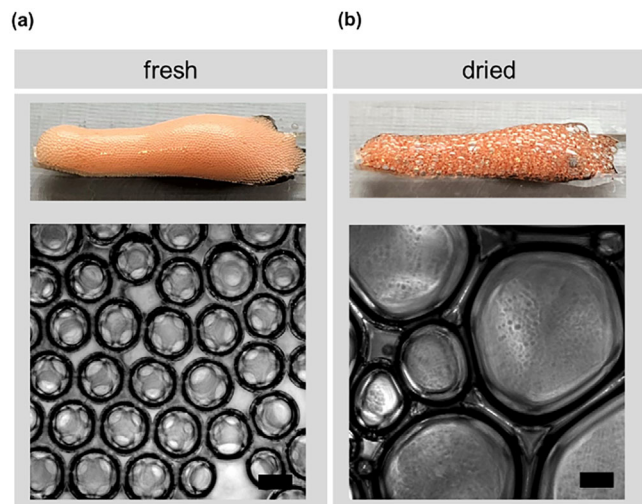


FIGURE 4 | Initial preparation and characterization of cytochrome c (CC) enzyme foams. (a) Formation of monodisperse bubbles by mixing an aqueous solution of self-assembling proteins (SC-CC-SC and STV-ST). Microscopy images recorded at 20 $\times$ magnification show the uniform bubble distribution; scale bar = 100 μ m. (b) After drying at 30 $^{\circ}$ C for 2 days, partial coalescence of bubbles and loss of the initially monodisperse structure occurred. Scale bar = 100 μ m.

dehydrogenase fused to SC (SC-ADH) and glucose dehydrogenase fused to ST (GDH-ST) (Figure 5a). This system provided a defined and quantitative platform to evaluate the coupling efficiency, foaming behavior, and stabilizing effect of the newly developed surface-active SC-Rsn2 fusion.

The addition of the surface-active Rsn2 in the form of the SC-Rsn2 fusion protein was expected to promote the attachment of the ADH/GDH hydrogel particles at the gas–liquid interface, where Rsn2 would shield the remaining proteins and stabilize the foam structure (Figure 5b). This interfacial localization should, in principle, enhance the overall wet-foam stability and suppress bubble coalescence. However, both the standard ADH/GDH foams and those containing 10% SC-Rsn2 showed a similar decrease in foam height over time, indicating that no significant stabilization was achieved (Figure 5c). Fluorescence microscopy using GFP-labeled enzyme foams allowed visualization of the pore structures at different drying stages (Figure 5d, Figure S11). Automated image analysis of pore diameters and lamella widths [85] confirmed that the incorporation of SC-Rsn2 did not significantly affect foam morphology or stability. This outcome is consistent with the known robustness of the ADH/GDH model system [62], which likely masked subtle interfacial effects. Nevertheless, the potential of SC-Rsn2 to stabilize less intrinsically stable systems warranted investigation in the context of the cytochrome c (CC) foams described above.

Incorporation of 10% SC-Rsn2 into the STV–CC APH foams did not improve bubble stability during or after formation (Figure S12). In the course of this study, however, an unexpected effect was identified: the reduced foam stability of the initial CC formulations likely correlated with the presence of glycerin in the storage buffer of the STV-ST component. Glycerin, known to influence interfacial tension, likely interfered with bubble stability as it becomes more concentrated during the evaporation

process. This observation suggests that highly polar additives can substantially alter the drying behavior and interfacial properties of the foam system. Consequently, while compounds such as polyols or sugars are frequently employed as protein stabilizers during drying, their incorporation into the present formulation may similarly affect foam structure and stability and therefore requires careful evaluation in future studies. After replacing the storage buffer with PB, the resulting enzyme foams exhibited the expected monodisperse bubble morphology immediately after generation (Figure 6a) and formed stable, solid red materials with a well-defined hexagonal pore structure after drying for 2 days at 30 $^{\circ}$ C (Figure 6b). The material displayed a uniform, open-pore architecture with well-defined hexagonal cavities, indicative of an extensive internal surface area and efficient accessibility of the enzyme throughout the foam matrix. The intense red coloration of the dried foams further confirmed the successful incorporation and structural integrity of the heme-containing CC within the material matrix.

Fragments of the resulting self-supporting, porous red CC foam were evaluated regarding the enzymatic activity using the model reaction from the chiral organosilicon compound **3** (Figure 6c). CC in the foam formulation showed comparable activity to the particle formulation (CC@AP) established earlier [63], but with lower yield as compared to unbound CC enzymes in homogeneous solution. The reduced activity observed for both immobilized formats relative to soluble CC is not unexpected, since immobilization can alter the local environment of the enzyme and may affect structural flexibility, substrate accessibility, or diffusion of reactants and products within the material. Moreover, a fraction of the enzyme molecules may become less accessible to substrates due to their incorporation into the dense protein network. Beyond immobilization-specific effects, previous studies on the CC enzyme system have shown that side reactions can contribute to catalyst deactivation during C–Si bond formation [64]. Such processes may be further promoted within immobilized materials owing to the high local enzyme concentrations present within the foam and particle matrices. Although the present study was not designed to distinguish between these contributions, future investigations combining structural characterization of immobilized CC variants, mass-transfer analyses, and detailed studies of catalyst deactivation kinetics can provide further insight into the origins of the reduced activity. Thus, these results demonstrate that cytochrome c remains catalytically active within the solid foam matrix, even after drying, and that this immobilization is equally suitable as the particle-based method established earlier [63], however circumventing the use of a commercial carrier material. The stable, open-pore morphology of the foam is expected to promote substrate accessibility and diffusion throughout the material, contributing to the sustained high enantioselectivity observed during catalysis. However, catalytic activity decreased over extended reaction times (Figure S13), which is consistent with the known gradual inactivation of the enzyme under the reaction conditions. Accordingly, these results support our previous observation that maintaining a high CC-to-substrate **1** ratio and short reaction times is essential for efficient and highly enantioselective silicon-carbon bond formation [64].

Previous studies on self-assembling enzyme foams demonstrated that prolonged drying can substantially improve material stability

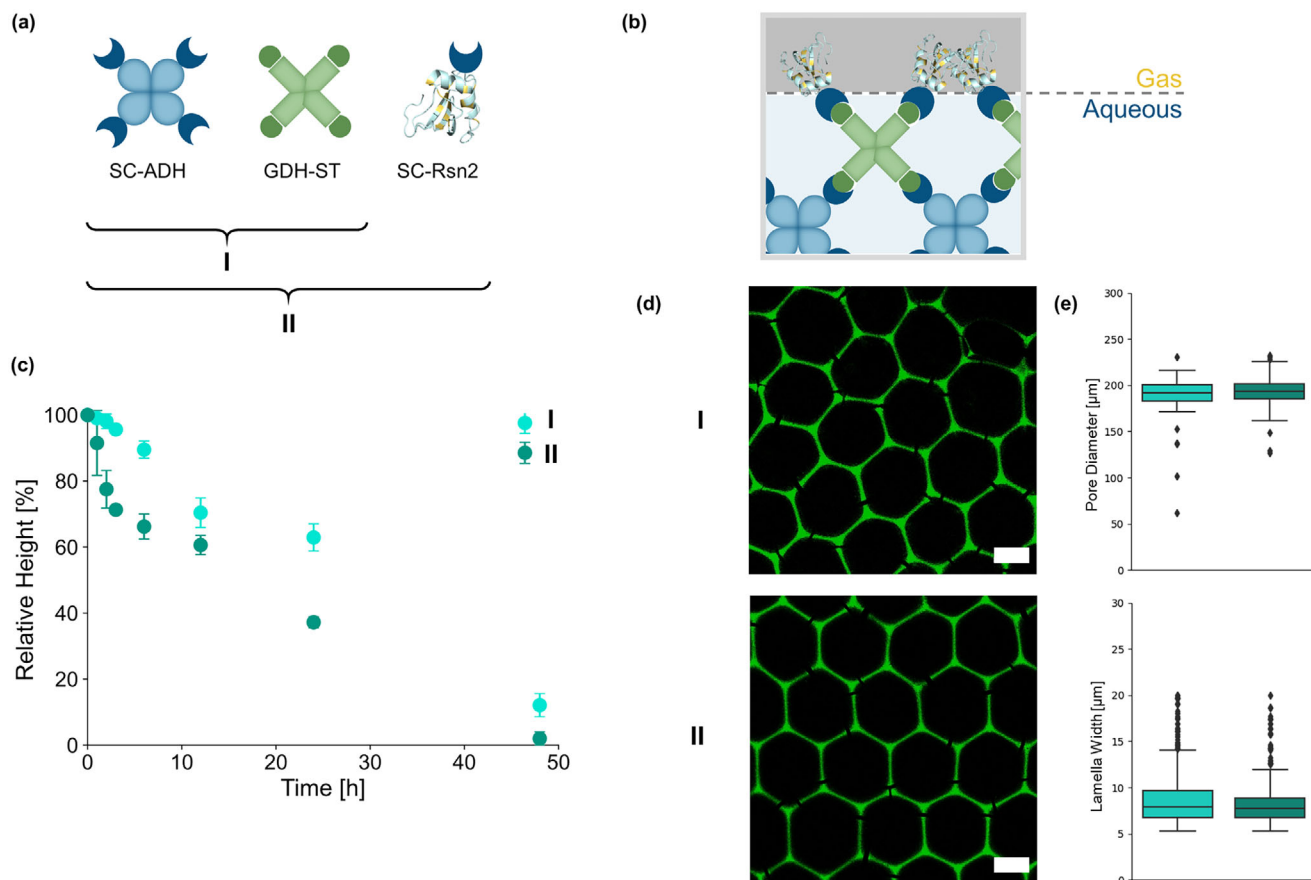


FIGURE 5 | Stabilization of ADH/GDH enzyme foams using the surface-active fusion protein SC-Rsn2. (a) Schematic representation of polymer formation based on GDH-ST and SC-ADH (I) and with additional incorporation of SC-Rsn2 (II, crystal structure PDB: 2WGO [82]). (b) Illustration of the localization of Rsn2 at the gas-liquid interface of the ADH/GDH foam, while the SC/ST-linked hydrogel components remain in the aqueous phase. (c) Foam height of wet ADH/GDH foam (I) and ADH/GDH foam containing SC-Rsn2 (II) as a function of time. (d) Fluorescence microscopy images (20× magnification) of dried ADH/GDH foams (I, II) labeled with 0.25% ST-GFP-ST after drying for 2 days at 30°C; scale bar = 100 μm. (e) Automated image analysis of the micrographs in (d) using a Python-based script to quantify pore diameters and lamella thickness distributions.

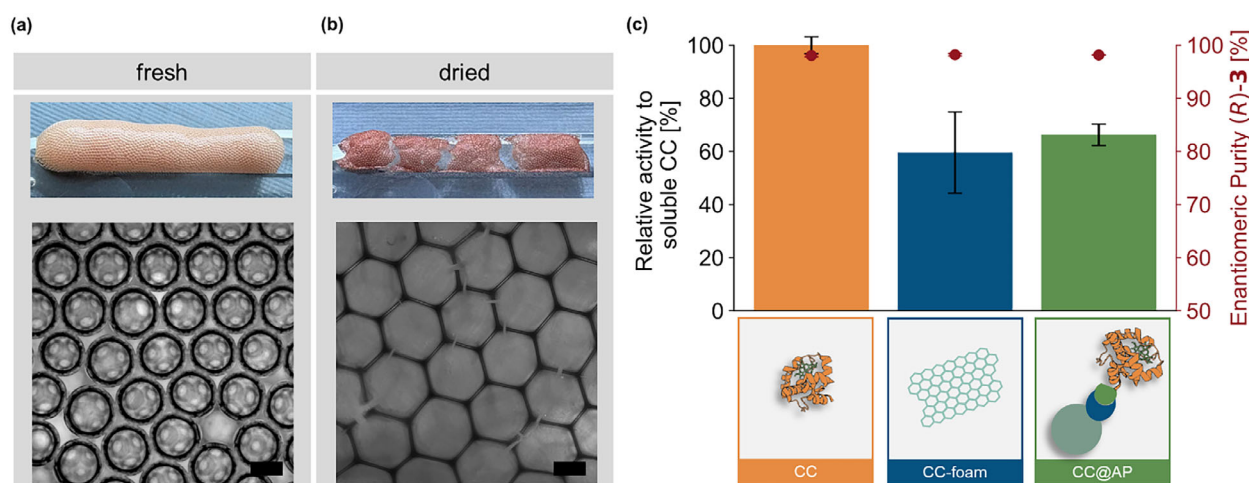


FIGURE 6 | Preparation and characterization of cytochrome c (CC) enzyme foams. (a) Formation of monodisperse bubbles by mixing an aqueous solution of self-assembling proteins (SC-CC-SC and STV-ST). Microscopy images recorded at 20× magnification show the uniform bubble distribution; scale bar = 100 μm. (b) After drying at 30°C for 2 days, a stable, red porous material with a hexagonal polyhedral pore structure is obtained. (c) Activity (bars) and enantiomeric excess of product **3** (circles) in batch reactions after 10 min using approximately 4.5 nmol (with regard to the CC amount) of soluble CC (orange), CC foam (blue) dried for 2 and CC@AP, performed with 10 mM NaDT, 5 mM **1**, and 10 mM **2** under anaerobic conditions. Error bars represent the standard deviation from duplicates.

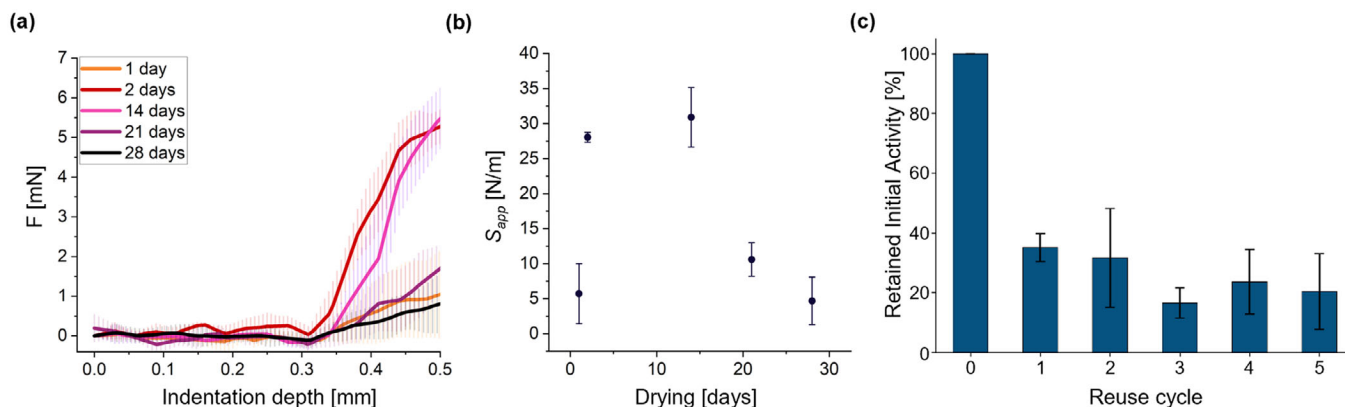


FIGURE 7 | Mechanical characterization of the CC foams at different drying times and during repeated batch catalysis. (a) Force versus indentation curves obtained for the foam dried for different intervals. (b) Apparent stiffness (S_{app}) obtained from the slopes of the curves shown in (a). (c) Retained activity after repetitive use of a CC foam over six consecutive reaction cycles. Error bars represent the standard deviation from at least three (a, b) and at least seven replicates (c).

and thereby enhance their suitability for reuse and continuous operation [61, 62]. We therefore investigated the influence of drying time on the structural and mechanical properties of the CC foams. Microscopic analysis revealed progressive changes in foam morphology during the first 2 days of drying, consistent with the formation of the characteristic polyhedral pore structure. Beyond this period, however, no further significant morphological changes were observed, and the foams retained a comparable pore architecture for drying times up to 28 days (Figure S14). These results indicate that the structural maturation of the foam is largely completed within the first 2 days, while prolonged drying primarily affects its mechanical properties. Mechanical characterization by indentation experiments revealed pronounced changes in the material response. Foams dried for 1, 2, and 14 days recovered their original morphology after indentation, indicating predominantly elastic behavior. In contrast, foams dried for 21 and 28 days exhibited irreversible deformation after unloading, suggesting a transition from an elastically recoverable state to a regime dominated by plastic yielding and/or cell-wall buckling. This behavior is reflected in both the average force-indentation curves (Figure 7a) and the corresponding apparent loading stiffness (S_{app}), determined from the slope of the approach curves between initial surface contact ($h \approx 0.3$ mm) and the maximum indentation depth ($h \approx 0.5$ mm) (Figure 7b). S_{app} increased substantially from 1 to 2 days of drying, remained largely constant up to 14 days, and subsequently decreased sharply after 21 and 28 days of drying. These observations suggest that initial dehydration stiffens the foam network, whereas prolonged drying alters the dominant deformation mechanism and promotes localized structural collapse during mechanical loading. To further quantify the elastic response, the apparent Young's modulus (E_{app}) was determined for all samples exhibiting complete morphological recovery using Sneddon's model (for details on the calculations see experimental section). Foams dried for 1, 2, and 14 days exhibited E_{app} values of 4.7 ± 3.5 , 23.1 ± 0.6 , and 25.5 ± 3.5 kPa, respectively. These values are in good agreement with reported Young's modulus ranges for porous protein-based scaffold materials and confirm the substantial stiffening of the foam network during the initial drying period [86].

The enhanced robustness obtained upon extended drying was further supported by subjecting the CC to continuous flow conditions. Consistent with previous observations for enzyme foams [61], foams dried for 28 days retained their characteristic hexagonal pore architecture after 24 h of continuous flow operation (Figure S15), demonstrating excellent structural stability. Based on these findings, foams dried for 28 days were selected for reusability studies in repeated batch reactions for the synthesis of organosilicon product 3 (Figure 7c). Upon contact with the reaction medium, the foams readily rehydrated but remained macroscopically intact (Figure S16). The material retained catalytic activity over five consecutive reaction cycles. An initial decrease in activity was observed after the first cycle, which is likely attributable to the removal of loosely attached foam fragments during the first complete exchange of the reaction solution. This interpretation is supported by Western blot analysis of the reaction supernatants, which revealed substantial amounts of foam-derived protein during the initial reaction cycle, whereas protein levels in the supernatants of the subsequent reuse cycles were below the detection limit (Figure S17). Thereafter, the remaining foam exhibited stable catalytic performance, maintaining more than 20% of the initial activity over five further reaction cycles. Together, these results demonstrate that prolonged drying substantially improves the operational stability of the material and enables the use of the CC foam as a reusable biocatalyst. The repeated-batch experiments additionally provide experimental support for sustained substrate accessibility within the foam matrix. Despite repeated replacement of the reaction solution, the material remained catalytically active and continuously converted freshly added substrate. These observations indicate that active enzyme molecules remain accessible within the interconnected pore network and that mass transport through the foam is maintained throughout prolonged operation. Combined, these results demonstrate that the CC foam combines catalytic activity, mechanical robustness, and reusability within a carrier-free material. The open and interconnected pore architecture facilitates efficient mass transport while maintaining high local catalyst concentrations. As such, the presented foam represents a promising platform for reusable biocatalysis and a carrier-free alternative to previously reported particle-supported CC immobilization approaches.

3 | Conclusion

In this work, we established a modular strategy for the development of carrier-free, self-supporting biocatalytic materials based on *R. marinus* cytochrome c (CC). Through specific crosslinking between SC-CC-SC and STV-ST, stable red enzyme foams were generated that combine high catalyst density with efficient mass transport. The foams retained structural integrity and catalytic activity even after drying, demonstrating that CC remains functional at extensive gas-liquid interfaces present within the dried, porous material. Although catalytic activity decreased during prolonged operation, consistent with the known gradual inactivation of the enzyme under the reaction conditions, the foam maintained excellent stereoselectivity and structural integrity. The resulting system effectively unites the advantages of immobilization with high enzyme loading and accessibility in a single biocatalytic framework.

Overall, the presented immobilization concept provides a viable alternative to particle-supported CC immobilization approaches [63, 64], and demonstrates that the foam fabrication protocol originally developed for multimeric enzymes [61] can be successfully extended to monomeric enzymes. Moreover, the STV-mediated crosslinking concept expands the method's versatility, enabling its application to other industrially relevant enzyme monomers and allowing the site-specific incorporation of biotinylated cofactors or other functional molecules via the strong STV-biotin interaction.

Together, these findings define a general and scalable route toward structurally defined, carrier-free biocatalytic materials with tunable architecture and reactivity, opening new perspectives for integrating enzymatic catalysis into sustainable chemical synthesis and continuous flow processes.

4 | Experimental Section

4.1 | Standard Protein Expression Procedure

Proteins were produced heterologous similar to previously described [56]. *E. coli* BL21(DE3) was transformed with the corresponding plasmid using heat shock transformation. *E. coli* cells harboring the plasmid were selected on LB/agar plates containing 100 µg/mL ampicillin overnight at 37°C. Liquid cultures of 100 mL TB medium containing ampicillin were generated from a single colony and cultured at 37°C and 180 rpm for 16 h. Two flasks with 2 L ampicillin containing TB-medium was inoculated with 40 mL overnight culture and incubated at 37°C and 180 rpm until an OD₆₀₀ of 1.1 was reached. The temperature was then lowered to 25°C, 500 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the cultures were incubated for an additional 20 h. The cells were harvested by centrifugation at 10,000 rcf and 4°C for 10 min, resuspended in 60 mL NP₁₀ buffer (500 mM NaCl, 50 mM NaH₂PO₄, 10 mM imidazole, pH 8.0) and stored at -80°C until further processing.

4.2 | Adjustments for CC Variants

The heterologous expression of CC variants was also performed similar to previously described [63] and deviations from the

expression above are indicated in the following. The respective plasmid for the CC variant was cotransformed with the cytochrome c maturation plasmid pEC86 [87] into *E. coli* BL21(DE3) using electroporation. LB/agar plates for the selection of the *E. coli* cells harboring both plasmids as well as the liquid TB cultures contained 30 µg mL⁻¹ chloramphenicol and 100 µg mL⁻¹ ampicillin. After an OD₆₀₀ of 1.1 was reached, the culture was then cooled on ice for 20 min. Expression was induced by adding 20 µM IPTG and 200 µM 5-aminolevulinic acid and the culture was incubated at 20°C and 180 rpm for 20 h.

4.3 | Adjustments for STV Variants

For the heterologous expression of STV variants, it was taken into account that they are located in inclusion bodies and therefore deviate from the above expression, which is indicated below. The respective plasmid was transformed into *E. coli* BL21(DE3)pLysE using electroporation. LB/agar plates for the selection of the *E. coli* cells harboring the plasmid as well as the liquid TB cultures contained 30 µg mL⁻¹ chloramphenicol and 100 µg mL⁻¹ ampicillin. Expression was induced by adding 500 µM IPTG and the culture was incubated at 37°C and 180 rpm for 20 h. The cells were harvested by centrifugation at 10,000 rcf and 4°C for 10 min, resuspended in 3 mL Tris A buffer (50 mM Tris-HCl, 500 mM sucrose, pH 8.0) per gram of pellet. After further centrifugation at 20,000 rcf and 4°C for 30 min, the cells were resuspended in the same amount of Tris B buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) and then lysed by ultrasonication. The inclusion bodies were sedimented by ultracentrifugation at 45,000 rcf and 4°C for 1 h and stored at -20°C until further processing. They were resuspended in 8 mL of 6 M guanidine hypochlorite solution (pH 1.5) per liter expression culture, stirred at 400 rpm and 4°C for 2 h and then centrifuged at 26,000 rcf and 4°C for 30 min. The supernatant containing unfolded STV was dropped into 250 mL PBS per liter expression culture at 4°C using a syringe pump with a flow rate of 0.5 mL min⁻¹ and allowed to rest overnight to refold the STV. Unfolded STV was removed by centrifugation at 20,000 rcf and 4°C for 30 min, the supernatant containing folded STV was stirred at 200 rpm and 4°C and ammonium sulfate was added up to a 40 % saturated solution. After 2 h incubation at 4°C, further ammonium sulfate was added up to a 70 % saturated solution that was then stirred at 200 rpm for 1 h and rested overnight at 4°C. The precipitated STV was centrifuged at 12,000 rcf and 4°C for 30 min, resuspended in 30 mL PBS (11.5 mM sodium phosphate, 50 mM NaCl, pH 7.3) and injected into a 10,000 MWCO dialysis cassette (Slide-A-Lyzer, Thermo Scientific) placed in ammonium carbonate buffer (50 mM ammonium carbonate, 0.5 M NaCl, pH 11.0) overnight at 300 rpm and 4°C.

4.4 | Protein Purification via His₆

Proteins tagged with his₆ were purified by fast protein liquid chromatography (FPLC) using its affinity to Ni, as previously described [63]. The cell suspension was thawed at 25° and subsequently incubated with DNaseI and lysozyme (AppliChem) for 30 min at room temperature. After ultrasonication, cell debris was removed using centrifugation at 45,000 rcf and 4°C for 1 h and the protein rich supernatant filtered through a 0.45 µm PVDF

membrane (Durapore, Steriflip, Millipore). The lysate was applied on a Ni-column (5 mL HisTrap FF, cytiva) installed in an FPLC system (Äkta pure, GE Healthcare). The column was first washed with a mixture of 2% NP₁500 (500 mM NaCl, 50 mM NaH₂PO₄, 500 mM imidazole, pH 8.0) and 98% NP₁10 and subsequently the protein was eluted using a linear gradient from 2% NP₁500 and 98% NP₁10 to 100% NP₁500 over 10 column volumes. The collected fractions containing the purified protein were combined and the buffer exchanged to PB (50 mM sodium phosphate, pH 7.4) for the CC variants, KP_i buffer (100 mM potassium phosphate, 1 mM MgCl₂, pH 7.5) for SC-ADH and GDH-ST or PBS for the other proteins using 5000 or 10,000 MWCO concentrators (Vivaspin 20, Sartorius). The concentrated protein solution was aliquoted, frozen in liquid nitrogen and stored at –80°C until used.

4.5 | Protein Purification via Biotin Affinity

After dialysis, STV variant was centrifuged at 45,000 rcf and 4°C for 1 h, filtered through a 0.45 µm PVDF membrane (Durapore, Steriflip, Millipore) and was applied on a column packed with Iminobiotin (IBA Lifesciences) installed in an FPLC system (Äkta Prime Plus, GE Healthcare). The column was first washed with ammonium carbonate buffer and then with a mixture of 2% ammonium acetate buffer (50 mM ammonium acetate, pH 4.0) and 98% ammonium carbonate buffer. The protein was eluted using 100% ammonium acetate buffer for 12 min. The collected fractions containing the purified protein were combined and the buffer exchanged to PBS using 5000 MWCO concentrators (Vivaspin 20, Sartorius). The concentrated protein solution was mixed with glycerin to a final concentration of 20% (v/v) for freezing. For foam preparation, buffer was exchanged to PB using 5000 MWCO concentrators (Vivaspin 500, Sartorius).

4.6 | Enzymatic Reactions

The activity of soluble or immobilized CC variants regarding the formation of silicon–carbon bonds was studied as previously described [63]. In a total volume of 400 µL of anaerobic PB or HEPES buffer (50 mM HEPES, pH 7.4) containing 5% MeCN, 5 µM of soluble or immobilized CC variant were mixed with 10 mM sodium dithionite, 10 or 5 mM ethyl 2-diazopropanoate (1) and 10 mM phenyl dimethylsilane (2). The components were combined in an anaerobic vinyl chamber and incubated anaerobically at 700 rpm and 25°C on a thermoshaker. The reaction was stopped by adding cyclohexane and analyzed by GC or chiral HPLC.

4.7 | Repeated Batch Experiments

Each reaction cycle was performed for 10 min using a 0.2 to 0.5 mg foam piece 400 mL reaction containing 10 mM substrate 1 and 2. After each cycle, the reaction solution was removed and analyzed. 400 mL of fresh substrate solution was added and a sample was taken at 0 min to account for any remaining product from the previous step. To account for the differences in foam mass all activities in the repeated batches were calculated relative to the respective initial activity of the individual replicate.

4.8 | Preparation of Nanoscale Hydrogel Particles

1 mM solutions of the hydrogel forming proteins were mixed in a ratio so that there were equal quantities of SC and ST units present.

4.9 | Dynamic Light Scattering (DLS)

Time-dependent increase of hydrodynamic diameter upon hydrogel formation was determined using a DLS device (Zetasizer Nano ZSP, Malvern Instruments), equipped with a He-Ne-Laser (633 nm), as previously described [56]. Before the measurement, the solutions of 1 mM or 0.1 mM hydrogel forming proteins were preincubated at 300°C and 500 rpm for 5 min in a thermoshaker. Subsequently, they were mixed in a UV cuvette in such a ratio that equal quantities of SC and ST units in a total volume of 100 µL were present. The average hydrodynamic diameter of the protein particles (Z-Average), calculated from auto-correlated light intensity data using the ZetaSizer Software, was recorded over 2 h with individual measurements every 30 or 60 s.

4.10 | Preparation of Foam

The formulation of enzyme foams was conducted as previously established [61]. A solution of self-assembling enzymes (1 mM, in KP_i buffer for ADH/GDH foams or PB for CC foams) containing equimolar amounts of SC and ST units was incubated on ice for 5 min. Subsequently, the protein solution was connected to the flow-focusing junction [88] and introduced using a microfluidic pressure pump (EZ flow, Fluigent) at 450 mbar and a nitrogen stream at 400 mbar. The resulting foam bubbles were collected and, if necessary, dried at 30°C until use. (STV-ST)₄ was transferred into PB using centrifugal concentrators (Vivaspin 500, 5 kDa, Sartorius).

4.11 | Particle Immobilized CC

ST-CC was immobilized on epoxy-agarose particles (AP) as previously described [63]. AP were first functionalized with SC [84], then blocked with ethanolamine solution before they were loaded with ST-CC for 1 h.

4.12 | Indenter Measurements

Mechanical characterization of the foams dried over varying times was performed via indentation analysis using a custom-built setup described in Lemke et al. [89]. Briefly, the device consists of a motorized XYZ platform for precise micro-positioning of the sample and a fixed flat-cylindrical plunger (1 mm diameter) coupled to a high-sensitivity force sensor. To remain strictly within the small-strain regime, mechanical analysis was restricted to the initial ~ 190 µm of indentation along the approach curve.

Raw force–displacement curves were corrected for plunger compliance, which was determined from the mean inverse slope of three force curves on a glass substrate. The apparent loading

stiffness (S_{app}) was extracted from the slope of the corrected force-indentation curves via linear fitting between initial surface contact and maximum indentation depth.

To determine the apparent Young's modulus (E_{app}) of the elastic samples while accounting for bottom-effect stiffening from the underlying rigid substrate on the ~ 5 mm thick samples, Sneddon's flat-punch model was modified using the finite-thickness correction factor derived by Cao et al. [90]:

$$E_{\text{app}} = \frac{1 - \nu^2}{2a \Pi\left(\frac{a}{t_0}, \nu\right)} S_{\text{app}}$$

where S_{app} is the apparent loading stiffness calculated from the slopes of the force-indentation curves, a is the plunger radius, t_0 is the uncompressed foam thickness, and ν is the Poisson ratio. The Poisson ratio was set to $\nu = 0.3$, a broadly accepted mean estimate for foams, falling well within the experimental range of 0.15–0.45 reported for some biological dry foams by Zhaohui et al. [91].

The dimensionless finite-thickness correction factor $\Pi = 1.105$ was determined by multiplying the theoretical scaling factor $f = 1.028$ ($\frac{a}{t_0} = 0.1$, $\nu = 0.3$) by the graphical finite-element solution ($\frac{\Pi}{f} = 1.075$ from Cao et al. [90]). Interfacial friction was neglected as ν was assumed to be < 0.45 .

Author Contributions

Marc F. Munker: investigation, formal analysis, data curation, writing – review and editing, writing – original draft, visualization, validation, methodology, software, conceptualization. **Annika J. Weber:** conceptualization, investigation, funding acquisition, writing – original draft, writing – review and editing, visualization, validation, methodology, software, formal analysis, data curation. **Madleen Richter:** writing – review and editing, visualization, methodology, validation, formal analysis, investigation, data curation. **Jennifer Kühne:** writing – review and editing, visualization, data curation, methodology. **Carmen M. Dominguez:** visualization, writing – review and editing, data curation, formal analysis. **Christof M. Niemeyer:** conceptualization, investigation, funding acquisition, writing – review and editing, supervision, writing – original draft. **Kersten S. Rabe:** conceptualization, investigation, funding acquisition, writing – original draft, writing – review and editing, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest. A related patent was filed including KSR and CMN as inventors.

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Supporting Information

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

Supplementary Figures and further experimental details on plasmid construction, protein analysis, activity determination as well as methods relevant for data presented in the Supporting Information are given there. The authors have cited additional references within the Supporting Information [92–104].

Supporting File 1: chem71776-sup-0001-SuppMat.pdf