Engineered Living Materials Based on Adhesin-Mediated Trapping of Programmable Cells


ABSTRACT: Engineered living materials have the potential for wide-ranging applications such as biosensing and treatment of diseases. Programmable materials provide the functional basis for living materials; however, their release into the environment raises numerous biosafety concerns. Current designs that limit the release of genetically engineered cells typically involve the fabrication of multilayer hybrid materials with submicrometer porous matrices. Nevertheless, the stringent physical barriers limit the diffusion of macromolecules and therefore the repertoire of molecules available for actuation in response to communication signals between cells and their environment. Here, we engineer a novel living material entitled “Platform for Adhesin-mediated Trapping of Cells in Hydrogels” (PATCH). This technology is based on engineered E. coli that displays an adhesion protein derived from an Antarctic bacterium with a high affinity for glucose. The adhesion stably anchors E. coli in dextran-based hydrogels with large pore diameters (10–100 μm) and reduces the leakage of bacteria into the environment by up to 100-fold. As an application of PATCH, we engineered E. coli to secrete the bacteriocin lysostaphin which specifically kills Staphylococcus aureus with low probability of raising antibiotic resistance. We demonstrated that living materials containing this lysostaphin-secreting E. coli inhibit the growth of S. aureus, including the strain resistant to methicillin (MRSA). Our tunable platform allows stable integration of programmable cells in dextran-based hydrogels without compromising free diffusion of macromolecules and could have potential applications in biotechnology and biomedicine.

Synthetic biology aims to design programmable cells that combine sensing and molecular computing operations with on-demand production of therapeutic relevant proteins.1–4 Engineered living materials (ELMs) integrate genetically engineered cells with free-standing materials and represent a new class of environmentally responsive living devices with designer physicochemical and material properties.5–8 Ideally, ELMs provide mechanical robustness to engineered cells, prevent their migration to the environment, and allow cells to be viable for extended periods of time. As it has been difficult to efficiently immobilize genetically modified microorganisms (GMMs) in ELMs, their release into the environments poses a major challenge for future synthetic biology applications.9,10 To date, strategies for containing GMMs inside a living device are based on the physical confinement by multilayer materials.11–13 Hybrid micro-patterned devices that combine layers of elastomer and microporous hydrogel have enabled the exchange of information with the surrounding environment via diffusion of chemical inducers and their sensing by GMMs while displaying high mechanical resilience.11 Nevertheless, the low porosity of the physical barriers mitigating bacterial escape also significantly hampers the diffusion of macromolecules, reducing the repertoire of synthetic biology applications to biosensing and release of small therapeutic molecules.

Programmable interactions between GMMs and their surroundings have recently gained interest as the design of multicellular structures such as engineered biofilms enables a
higher control over shape and functionality of ELMs. \textsuperscript{14,15} Inducible secretion of fibrous proteins drives the emergence of molecular architectures supporting the artificial biofilms. Interestingly, such biofilms are self-regenerating and are able to perform advanced tasks such as multistep enzymatic bioremediation, and are viable over weeks. \textsuperscript{14–17}

Inspired by this strategy, we propose to leverage the formation of programmable interactions between surface-displayed proteins on genetically engineered cells and hydrogel constituents to ensure cell containment within a living material while simultaneously allowing production and delivery of large therapeutic molecules. The “Platform for Adhesin-mediated Trapping of Cells in Hydrogels” (PATCH) is based on engineered \textit{E. coli} expressing a recombinant adhesion protein that stably anchors the cells to a hydrogel matrix, avoiding entrapment by stringent physical barriers (Figure 1a). Bacterial adhesins are modular cell-surface proteins that bind different ligands in a highly specific manner. While strategies for programmable cell–cell interactions via adhesins have been reported,\textsuperscript{6,19} methods that allow adhesin-mediated cell–material interactions have not been described. We engineer \textit{E. coli} bacteria to surface-display a Ca\textsuperscript{2+}-dependent glucose-binding adhesin which has strong interactions with dextran-based hydrogels (Figure 1b,c for details). The engineered adhesin, referred to as \textit{Mp}A, is derived from the \textit{Marinomonas primoryensis} ice-binding protein (\textit{Mp}IBP) which helps these bacteria to assemble into multispecies biofilms on ice.\textsuperscript{20–22} Compared to conventional living material designs, our hydrogel-based material has large pores with diameters ranging from 10 to 100 μm, which substantially improves the diffusion of macromolecules through the matrix. Furthermore, our results reveal efficient containment of adhesin displaying bacteria with a retention factor of up to 100-fold higher compared to bacteria that lack the adhesin. PATCH may help simplify the design of ELMs and could reduce the use of multimaterial or multilayer devices aimed at immobilizing GMMs.

Hydrogels with antimicrobial activity have become a major focus in biomedical research due to their unique qualities such as high biocompatibility and ease of drug loading and release.\textsuperscript{23,24} As an application of PATCH, we anchor \textit{E. coli} bacteria to surface-display a Ca\textsuperscript{2+}-dependent glucose-binding adhesin which has strong interactions with dextran-based hydrogels (Figure 1d,e) and show successful inhibition of \textit{S. aureus} growth including a methicillin-resistant (MRSA) strain. Due to the rising incidence of resistance, MRSA is becoming increasingly difficult to treat by conventional antibiotics.\textsuperscript{25} In the United States alone, MRSA causes approximately 11 000 deaths annually.\textsuperscript{25} Since lysostaphin functions by cleaving the pentaglycine components of the cell wall of \textit{S. aureus} including MRSA,\textsuperscript{26} it is highly specific in killing the bacterium. Importantly, lysostaphin is an enzyme that differs from small-molecule antibiotics, and is effective against antibiotic resistant infections.\textsuperscript{27} Taken together, our study demonstrates PATCH as a programmable living material foundry with potential applications in biosensing and controlled delivery of therapeutic proteins.

\section{RESULTS AND DISCUSSION}

\subsection*{Synthesis and Characterization of Dextran-Based Hydrogels.} Dextran-based hydrogels are relatively simple to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} The glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} The glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} The glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to synthesize, robust in various shapes and highly biocompatible.\textsuperscript{28–30} It is important to note that the glucose-based nature of the biopolymer makes it easy to sy
MpA allows E. coli to bind to the dextran matrix, thereby retaining the bacteria in the hydrogel (boundary marked by blue dashed lines). The engineered E. coli inside the hydrogel can secrete lysostaphin (small red circles), which diffuses freely to the exterior environment to inhibit the growth of S. aureus (gold spheres), including a methicillin-resistant strain (MRSA). Gold spheres with dashed boundaries indicate dead S. aureus cells killed by lysostaphin.

dextran an ideal material for anchoring E. coli expressing MpA. The dextran (500 kDa) polymer was functionalized by methacrylation to yield methacrylated dextran (Dex-MA) (Figure S1.1–1.2), which was subsequently cross-linked into a covalent hydrogel via radical polymerization. The resulting hydrogel is easy to handle (i.e., not brittle or very viscous), and can be adapted into various shapes tailored for different applications. Further analysis of the wash solution of these hydrogels using NMR and FT-IR spectroscopy demonstrated that negligible concentrations of unreacted monomers are released from the hydrogel (Figure S1.3 Figure S2), which makes it suitable for keeping bacteria viable and likely safe for various medical applications. Scanning electron microscope (SEM) analysis showed that the dextran-based hydrogel has an overall microporous structure with pore diameters ranging from 10 to 100 μm (Figure S3). These large pores would allow unrestricted diffusion of macromolecules, as well as the propagation of native E. coli cells (2–5 μm), resulting in the migration of bacteria to the outside of hydrogel (side infra).

Cell-Surface Display of MpA. To stably anchor E. coli to dextran-based hydrogel, we designed a new adhesion protein, MpA, that binds glucose with high affinity and can be surface-displayed on E. coli. MpA is derived from the giant adhesin MpiBP (1.5 MDa) (Figure 2a) found on the cell surface of the Antarctic Gram-negative bacterium, Marinomonas primoryensis. MpiBP binds its bacterium to ice and facilitates the formation of symbiotic biofilms with other microorganisms.18 MpiBP has a C-terminal signal sequence enabling its secretion by the Type I Secretion System (T1SS) specific to M. primoryensis (Figure 2a).21 At the N terminus, MpiBP contains an “anchor module” that helps retain the protein to the cell surface by plugging the T1SS outer-membrane pore.21,22,32–34 The protein further consists of 120 identical 104-aa tandem repeats that serve to project a set of ligand-binding modules away from the cell surface to interact with their target molecules, including various carbohydrates, proteins, and ice. The sugar-binding domain (SBD) of MpiBP binds to glucose in a Ca2+-dependent manner,21,32,33 and is used in this study to allow engineered bacteria to bind to dextran-based hydrogels.

The extremely large molecular weight of MpiBP would likely prevent its use as a modular protein for cell-surface display in E. coli. We therefore introduced several modifications resulting in an engineered MpiBP-based variant, MpA, optimized for cell-surface display. First, we reduced the number of central MpiBP repeats from 120 to 15, lowering the molecular weight by approximately 5-fold (to 330 kDa; Figure 2b). These 15 tandem extender domains can project the sugar-binding domain (SBD) approximately 90 nm away from the cell surface.18 Next, the C-terminal domain (181 aa) of MpiBP was replaced by a 23 kDa C-terminal sequence of the E. coli hemolysin protein A (HlyAc) (Figure 2b), which enables translocation of the protein to the cell surface via the Type I secretion pathway (T1SS) specific to E. coli.36–39 The engineered mpa genes were placed in a pET24a vector. Since the genome of E. coli BLR (DE3) does not contain the T1SS machinery genes hlyb and hlyd required for secretion (pSTV28-hlyb/d; Figure 1b),39 a pSTV28 vector carrying

![Figure 2](https://dx.doi.org/10.1021/acssynbio.9b00404)
these two genes was cotransformed with the adhesin-pET24a vector (Figure 1b). We refer to bacterial cells cotransformed with these plasmids as $MpA^+$ cells and will use this notation throughout this article for other variants. Additional variants were created by replacing the N-terminal domains of $MpA$ with a fluorescent protein ($MpA$-mRuby2$^+$), which was used to validate the localization of the adhesin by confocal microscopy (Figure 2b). It has been reported that fluorescent proteins fold rapidly in the bacterial cytosol before being secreted by T1SS, and their large size prevents full passage through the outer-membrane pore of the secretion system. Thus, mRuby2 serves as an “anchor” to hold $MpA$ to the cell surface, while the remaining parts of the protein protrude into the extracellular environments. As a control, we prepared $MpA^-$ cells by cotransformation with a pET24a plasmid that only encodes for HlyAc (pET24a-hlyac) and the pSTV28_hlyb/d vector. As detailed later in the paper, we also created $MpA^+$ cells capable of secreting the bacteriocin lysostaphin to target Staphylococcus aureus. The resulting variant is referred to as $MpA^+$/lyso$^-$.  

Next, a combination of flow cytometry and confocal microscopy was used to validate the expression of $MpA$ on the outer surface of $E. coli$. $MpA^+$/lyso$^+$ (cells capable of expressing both $MpA$ and lysostaphin proteins, Figure 1d, details below) and $MpA^+$/lyso$^+$ control cells were incubated with rabbit antisera raised against the central repeats of $MpA$. After unbound primary antibodies were removed by washing, cells were further incubated with a fluorescently labeled (Alexa 488) secondary antibody (Figure 2c, top schematics). As expected, $MpA^+$/lyso$^+$ cells (Figure 2c, red histogram) showed a median fluorescence level approximately 5-fold higher than that of the $MpA^+$/lyso$^-$ cells (Figure 2c, gray histogram), validating the surface localization of the adhesin. Confocal microscopy studies using the $MpA$-mRuby2$^+$ variant revealed successful overexpression of the protein as evident by the evenly distributed red fluorescence inside of the cells. The surface display of this protein is demonstrated by the green immuno-fluorescence on the exterior of the cells. As the primary antibody targets the $MpA$ extender region consisting of 15 identical 104-aa repeats, it is likely that multiple antibodies bind to an individual adhesin protruding from the T1SS outer-membrane pore. This results in amplification of green fluorescence from the Alex488 secondary antibodies, which appears as green clusters on the cell surface (Figure 2d). These data are in agreement with the flow cytometry data. In summary, we designed a novel MipBP-based protein for cell-surface display in $E. coli$ and characterized the expression and localization of this protein using flow cytometry and confocal microscopy.

$MpA$ Efficiently Retains $E. coli$ inside Dextran-Based Hydrogels. To validate if the surface expression of $MpA$ retains $E. coli$ inside dextran-based hydrogels, we designed two experiments to quantify bacterial migration from the hydrogel matrix into liquid medium. Leakage of $MpA^+$ (orange) from a hydrogel cube was assessed by counting the colony forming units (CFUs) present in the LB medium surrounding the hydrogel, and compared with the leakage obtained from $MpA^-$ cells (blue, negative control). (b) Bacterial migration was quantified immediately after bacterial loading (initial leakage) and over the following day. Right panel: representative images of plates at the same dilution are shown to illustrate the difference in migration between $MpA^+$ and $MpA^-$ samples after 24 h (Day 1). Dots on the bar graph represent the results of individual experiments. (c) Scanning electron microscopy images obtained from the dextran-based hydrogel inoculated with the $MpA^+ E. coli$ inside of the bacterial loading chamber (cross-section). (d) Zoomed-in view of an area of panel c, showing a bacterial microcolony of $MpA^+$ formed on the lamina of the hydrogel matrix. Scale bars (green) in panels c and d indicate 10 $\mu$m. (e) Representative images showing the migration of $E. coli$ through the matrix of a disk-shaped hydrogel, which was assessed by estimating the surface area colonized by the bacteria ($MpA^+$/lyso$^-$ or $MpA^+$/lyso$^+$) on the outside of the hydrogel disk over time. Arrowheads indicate the presence of nascent colonies of $E. coli$ ($MpA^+$/lyso in blue) and ($MpA^+$/lyso$^+$ in orange). Scale bar represents 2 $mm$. (f,g,h) Quantification of the time-dependent migration of engineered $E. coli$ ($MpA^+$/lyso$^+$ in blue and $MpA^+$/lyso$^+$ in orange) through the hydrogel matrix. Average area and standard deviation of the bacteria-colonized regions outside of the hydrogel were calculated from replicates of that in panel e over 72 h.
portion of loaded bacteria directly flowing through the gel without binding to dextran. After unbound cells were removed by refreshing the medium, very few \( \text{Mp}^+ \) bacteria were present in the medium outside of the hydrogel in the next 24 h (approx. 30 CFUs). In sharp contrast, up to 100-fold \( \text{Mp}^- \) cells were present in the medium (approx. \( 2 \times 10^3 \) CFUs) (Figure 3b). SEM analysis was performed to gain visual insight

\[(n = 4). (f) \text{Experiments performed in the presence of both inducing agents, IPTG for adhesin surface display, and arabinose for lysostaphin expression (Ara).} (g) \text{Experiments performed in the presence of IPTG, but in the absence of Ara (}n = 3). (h) \text{Experiments performed in the absence of IPTG or Ara (}n = 3). \]

Figure 3. continued

Figure 4. PATCH with Anti-\( S. \text{ aureus} \) activity. (a) Schematic of the adapted Kirby-Bauer test for assessing the anti-\( S. \text{ aureus} \) activity of lysostaphin-secreting \( E. \text{ coli} \) as an application of PATCH. (b) Evaluation of bactericidal activity of the cell-free media collected from \( \text{Mp}^-/\text{lyso}^-, \text{Mp}^-/\text{lyso}^+, \text{Mp}^+/\text{lyso}^- \) and \( \text{Mp}^+/\text{lyso}^+ \) cells against \( S. \text{ aureus} \) and \( S. \text{ agalactiae} \). White arrows indicate where the samples were applied. Black arrows indicate the target bacteria streaked on the plates. Growth inhibition zones are marked by a red arrow. (c) Evaluation of bactericidal activity of \( \text{Mp}^-/\text{lyso}^-, \text{Mp}^-/\text{lyso}^+, \text{Mp}^+/\text{lyso}^- \) and \( \text{Mp}^+/\text{lyso}^+ \) (no lysostaphin-producing capability) \( E. \text{ coli} \) cells against \( \text{Methicillin-resistant S. aureus} \) (MRSA) and \( S. \text{ agalactiae} \). Images were captured after an incubation time of approximately 48 h. (d) Evaluation of bactericidal activity of the living hydrogels with engineered \( \text{Mp}^+/\text{lyso}^+ \) \( E. \text{ coli} \) cells spotted to the centers of the hydrogel disks, which were placed onto Mueller-Hinton agar plates streaked with MRSA. The images were captured after an incubation period of 96 h. The adhesin- and lysostaphin-functionalities were controlled by induction by IPTG and/or Arabinose (Ara), respectively. White arrowheads point to the edge of the hydrogel disks as outlined by thin white circles.
into how bacteria interacted with the hydrogel. We observed that Mpa\(^+\) bacteria formed thin layers of microcolonies on the lamina of the hydrogel due to their strong interaction with the gel matrix (Figure 3c,d). In contrast, Mpa\(^-\) bacteria showed a significantly sparser distribution (Figure S5a–d), indicating a weaker interaction with the hydrogel, which was consistent with the results obtained from the leakage experiments. In a different experiment we assessed the capability of Mpa\(^+\) in retaining the E. coli to the dextran-based matrix, by comparing the relative affinity of Mpa\(^+\) and Mpa\(^-\) cells to small hydrogel cylinders (~100 mm\(^3\)). Hydrogels were preincubated with either Mpa\(^+\) or Mpa\(^-\) overnight, and then extensively washed to remove unbound bacteria. Mpa\(^+\) or Mpa\(^-\) that remained bound to the hydrogels were released into a solution by homogenization and were quantified by CFU counting. As expected, hydrogels retained roughly 4-fold more Mpa\(^+\) bacteria compared to those that contained Mpa\(^-\) (Figure S5e), demonstrating the significantly stronger interaction between the bacteria and the hydrogel caused by Mpa\(^+\) expression.

Next, we tested whether surface display of Mpa\(^+\) also inhibits migration of bacteria into a surrounding solid medium. Small disk-shaped dextran hydrogels (thickness, 0.75 mm; diameter, 8 mm) were fabricated. The hydrogel disk was then placed on a LB-agar plate, and Mpa\(^+\)/lyso\(^+\) cells or Mpa\(^-\)/lyso\(^+\) control (1 \(\mu\)L) was loaded to the center of the disk. Bacterial migration from the center of the hydrogel matrix was monitored for up to 72 h (Figure 3e). We quantified the average colonized area outside the hydrogel disk based on four replicates. We observed that after roughly 10 h, Mpa\(^+\)/lyso\(^+\) cells started to migrate to the outside of the hydrogel, whereas Mpa\(^-\)/lyso\(^+\) cells were retained for at least another 25 h (Figure 3f). After 72 h Mpa\(^+\)/lyso\(^+\) cells colonized a surface 8-fold larger compared to Mpa\(^-\)/lyso\(^+\) cells (Figure 3g). Furthermore, uninduced Mpa\(^+\)/lyso\(^+\) cells did not show significant differences in migratory behavior compared to either induced or uninduced Mpa\(^+\)/lyso\(^+\) control (-IPTG, Figure 3f–h). The expression and secretion of an additional protein, lysostaphin, which was induced by arabinose (details below, Figure 1d), did not appear to hamper Mpa\(^+\)-mediated retention of the engineered E. coli to the hydrogel matrix (Figure 3f). These results further consolidated our findings that Mpa\(^+\) surface display can substantially enhance the retention of bacteria inside dextran-based hydrogels through its sugar-binding capability.

**Application of PATCH - Living Material Engineered to Kill MRSA.** Having developed PATCH, we next assessed whether Mpa\(^+\) bacteria can be used to deliver functional proteins in situ. We focused on engineering E. coli to secrete lysostaphin, a bacteriocin produced by Staphylococcus simulans to kill S. aureus.\(^{26}\) The full-length lysostaphin is a 50-kDa preproenzyme with a modular domain architecture including a 36-aa N-terminal signal peptide followed by a region comprising 15 tandem repeats of 13-aa, and the peptidase and cell-wall-targeting (CWT) domains at the C terminus.\(^{41}\) After its secretion from S. simulans, the signal peptide and the repetitive region of prepro-lysostaphin are sequentially cleaved after secretion. Given the capability of lysostaphin to cleave pentaglycine and the presence of two tetraglycine motifs linking lysostaphin and HlyAc, it is possible that the fusion protein underwent site-specific autolysis. Nevertheless, this would have no negative impact on the enzymatic capability of lysostaphin as it frees up its catalytic domains.

We quantified the efficacy of the secreted lysostaphin-HlyAc against S. aureus, using an adapted version of Kirby-Bauer (disk diffusion) test (Figure 4a).\(^{44}\) We applied cell-free medium of Mpa\(^+\)/lyso\(^+\) bacteria to Mueller-Hinton agar plates pre-streaked with S. aureus. After an overnight incubation period, clear round exclusion zones formed where the medium was applied, indicating the effective inhibition of S.aureus growth (Figure 4b). In contrast, cell-free medium from the Mpa\(^-\) cells showed no activity against S. aureus, indicating that growth inhibition was solely caused by lysostaphin secretion. Mpa\(^+\)/lyso\(^+\) cells were unable to inhibit the growth of a different Gram-positive pathogen, Streptococcus agalactiae, demonstrating the specificity of lysostaphin. To further consolidate this result, we spotted living Mpa\(^+\)/lyso\(^+\) cell cultures onto a Mueller-Hinton agar plate streaked with the notorious methicillin-resistant Staphylococcus aureus (MRSA), which is a major cause for hospital-acquired infections worldwide.\(^{45,46}\) Strikingly, we observed clear halo-like exclusion zones surrounding the Mpa\(^+\)/lyso\(^+\) cells, indicating the inhibition of MRSA growth (Figure 4c). These results demonstrate that sufficient amount of functional lysostaphin was secreted even though the Mpa\(^+\) adhesion and lysostaphin compete for T1SS ducts. In sharp contrast, no such inhibition zones separated the E. coli cells without lysostaphin-producing capability (Mpa\(^+\) cells) and MRSA. Consistent with the results above, Mpa\(^+\)/lyso\(^+\) cells could not kill S. agalactiae. These results demonstrated the high specificity and promising potential of
in situ lysostaphin secretion for treating antibiotic-resistant S. aureus-related infections.

After demonstrating that MpA'/lyso' cells can secrete functional lysostaphin in situ, we next investigated whether PATCH could kill MRSA, without leakage of GMMs. This would be a key step toward demonstrating the feasibility of this novel platform for different applications. As anticipated, very few E. coli cells induced with MpA'/lyso' migrated outside of the hydrogel disks into the surrounding Mueller-Hinton agar medium after 96 h (Figure 4d). Remarkably, the bacteria-loaded hydrogel disks are also effective in killing MRSA, as exclusion zones developed around the periphery of the devices. This demonstrates free diffusion of functional lysostaphin through the hydrogel matrix to the exterior environments. In contrast, when MpA expression was not induced (IPTG-), MpA'/lyso' cells were not restrained to the hydrogel, resulting in their migration into the exterior agar medium (Figure 4d, two panels on the right). When only lysostaphin expression was induced (IPTG+, Ara+), the inhibition zone significantly larger than when both MpA and lysostaphin were induced (IPTG+, Ara+). We reason that since the adhesin plugs a significant fraction of T1SS machineries, halting MpA production resulted in higher secretion levels of lysostaphin. Furthermore, a control experiment in which lysostaphin expression was not induced (IPTG-, Ara-) resulted in the absence of an inhibition zone, which validates the responsiveness of our system toward αrabinose induction (Figure 4d). Taken together, we have created a living hydrogel hosting adhesin-mediated self-retaining E. coli. As an application of this versatile platform, we demonstrated a prototype living material with anti-MRSA activity.

## CONCLUSIONS AND OUTLOOK

One challenge that impedes the deployment of living functional materials for biomedical applications outside the lab is the requirement of sustaining viable cells while restricting their escape from the device. To the best of our knowledge, this study shows the first example toward confinement of GMMs by actively implementing adhesive interactions between microorganisms and a fully synthetic material. The basis of PATCH is in contrast with conventional methods for engineering living materials, which typically rely on the presence of a physical barrier provided by submicrometer matrices of multilayer hybrid materials. Our work demonstrates one application of PATCH as a living material foundry for clearing pathogenic bacteria in situ production and secretion of lysostaphin.

PATCH presents a versatile toolbox that can be tailored to the need of various applications such as biosensing and smart drug delivery in a deployable “Sensel-lill” manner, which opens the door for a wide range of future research. Given the modularity of bacterial adhesins, PATCH can be easily tailored to other materials. Besides the SBD, another ligand-binding domain of MpA can be used to bind peptide-based materials. In addition, the E. coli pilus domain FimH has high specificity for mannose-based structures. Likewise, the E. coli pilus domain FimH has high specificity for mannose-based structures. Other constituents of PATCH such as gel porosity can be tuned to modulate the diffusion of various therapeutic compounds secreted by the bacteria. Identification of soluble S. aureus molecules capable of regulating the expression of lysostaphin would enhance the responsiveness and specificity of the current system. Implementing an alternative secretion system for lysostaphin might result in higher levels of in situ drug delivery. Crucially, integration of the expression systems into the genomes of the engineered microorganisms will help diminish the spreading of antibiotic resistant genes to pathogens via the plasmids. To address further safety concerns, we envision that the platform could be adapted to other microorganisms that previously demonstrated their innocuity for humans, and were used in other animal models for synthetic biology applications, such as E. coli Nissle 1917 strain (EcN) or Bacillus subtilis. As such, this work constitutes an important methodological improvement in the field of living functional materials and living material foundries, and could have implications for therapeutic applications.

## MATERIALS AND METHODS

**Gene Synthesis of MpA.** A gene encoding a smaller version of MpIBP (Genbank accession: ABL74378.1 and ABL74377.1) with only 15 central repeats was synthesized and purchased from GenScript (https://www.genscript.com). The gene was codon-optimized for expression in E. coli, and the central repeats were made as different as possible at the DNA level to reduce the chance of recombination. Next, the M. primoryensis-specific Type I Secretion System (T1SS) sequence near its C terminus (181 aa) was replaced by the E. coli T1SS sequence (last 213 aa of the Hemolysin A protein, HlyAc, also codon-optimized and synthesized by GenScript) to result in the MpA adhesin (Figure 2b). A fluorescent variant of MpA, MpA-mRuby2, was created by replacing the membrane anchor (412 aa at the N terminus) of the adhesin with mRuby2 (Figure 2b). Each MpA construct was placed between the Ndel and Xhol sites in a pET24a vector. Genes encoding two other machinery proteins (hlyb and hlyd) of E. coli T1SS were placed between the SacI and BamHI sites in a pSTV28 plasmid as described. The hlyb/d genes were PCR amplified from a pLGS57 plasmid kindly provided by Professor Dr. Peter Sebo (Institute of Microbiology of the Czech Academy of Sciences).

**Flow Cytometry.** Approximately 10⁶ of either induced MpA'/lyso' or MpA'/lyso' cells were collected, blocked with 1 mg/mL bovine serum albumin (Sigma-Aldrich) in a buffer containing 20 mM Tris-HCl pH 9, 150 mM NaCl, and 5 mM CaCl₂ (Buffer A), and incubated (1:100, 1 h) with rabbit antiserum raised against the central repeats of MpA. After washing with buffer A (2×), cells were incubated with a goat antirabbit secondary antibody (Alexa 633, Invitrogen; 1:100, 1 h) and then analyzed by a Fluorescence Activated Cell Sorter (Aria III, BD Sciences). The stained cells were gated based on forward and side scattering. At least 10 000 gated events were analyzed and plotted using the FlowJo software.

**Confocal Microscopy.** Cells were prepared in a similar way as those for flow cytometry experiments described above. MpA-mRuby2-expressing cells were collected, blocked with 1 mg/mL bovine serum albumin (Sigma-Aldrich) in Buffer A, and incubated (1:100, 1 h) with rabbit antiserum raised against the central repeats of MpA. After washing with buffer A (2×), cells were incubated with a goat antirabbit secondary antibody (Alexa 633, Invitrogen; 1:100, 1 h) and then analyzed by a Fluorescence Activated Cell Sorter (Aria III, BD Sciences). The stained cells were gated based on forward and side scattering. At least 10 000 gated events were analyzed and plotted using the FlowJo software.
Synthesis of Methacrylated Dextran. Dextran T500 (500 kDa, Pharmacosmos) (4 g, 24.7 mmol glucopyranose unit) was dissolved in 36 mL of dimethyl sulfoxide (DMSO) and degassed with argon for 15 min. After dissolving 800 mg of 4-dimethylaminopyridine (DMAP, Sigma-Aldrich), 2.4 mL of glycidyl methacrylate (GMA, Sigma-Aldrich) was added. The solution was stirred at room temperature for 44 h and the reaction was stopped by adding 6.6 mL of 1 M HCl to neutralize the DMAP. The reaction mixture was transferred to a SnakeSkin Dialysis bag (molecular weight cutoff: 10 kDa, Thermo scientific) and dialyzed against demineralized water at room temperature with changing water 3–4 times a day for 72 h. Next, the methacrylated dextran solution was lyophilized and stored at −30 °C before use. For NMR measurements, pharmaceutical-grade dextran 500 and methacrylated dextran samples were prepared in 10 mg/mL CDCl₃ and 1 mg/mL D₂O, respectively. The samples were measured on aBruker Avance 3 HD NanoBay spectrometer (1H NMR 400 MHz) at 23 °C. The degree of substitution, which is the percentage of methacrylated glucose monomers, was calculated with the formula (x/1.04y) × 100, where x stands for the average integral of the protons of the double bond, indicated with 22′ and 22″ in the spectrum, y stands for the integral of the anomeric proton, indicated with 4 in the spectrum, and 1.04 is the correction factor for the average 4% of α – 1, 3 linkages in dextran (Figure S1.2).

Dextran-Based Hydrogel Preparation. An aqueous solution of methacrylated dextran (5% w/v, 200 mg methacrylated dextran in 4 mL of H₂O) and ammonium persulfate (4.5 mg) was degassed with argon, stirred, and cooled in an ice bath for 15 min. From here on, all preparation was done at 4 °C in a cold room. After the addition of 5 µL of tetramethylethylenediamine (TEMED), the solution was mixed vigorously for 4−5 s and poured into the mold for hydrogel formation via freezing at −20 °C.

Assessment and Quantification of Unreacted Monomers Present in Hydrogel Wash Solutions by NMR and FT-IR. Samples of the wash solution were prepared by soaking a ready-to-use hydrogel (10 × 10 mm) in 5 mL of UP-mQ (ultrapure miliQ for analytical purposes). After 72 h the gel was removed, and the UP-mQ was freeze-dried in a 50 mL falcon tube. Subsequently, 800 µL of D₂O was added to the falcon tube and sonicated for 5 min. This solution was filtered through a syringe filter (0.2 µm) to remove residual dextran hydrogel that was still present in the sample. For NMR measurement, a sample (0.2% methanol as an internal standard) was measured on a Bruker Avance 3 HD NanoBay spectrometer (1H NMR 400 MHz) with a water suppression standard) was measured on a Bruker Avance 3 HD NanoBay spectrometer (1H NMR 400 MHz) at 23 °C. The liquid-state IR spectrum for the sample was recorded on a PerkinElmer Spectrum One spectrometer in a CaF₂ cell. The NMR and FT-IR experiments were performed at 25 °C.

Mold Fabrication for the Cubic Hydrogel. The device mold was fabricated in two steps. First, a poly(methyl methacrylate) (PMMA) mold was made using a CO₂ laser (see Figure S4a) and glued to a flat PMMA using an acrylic glue. Second, a PDMS prepolymer mixture was prepared in a 10:1 ratio (PDMS: curing agent) and poured over the mold. This was thermally cured in an oven at 65 °C for 2 h, and then the polymer was carefully peeled off from the mold (see Figure S4b). A side hole (θ = 2 mm) was made at the center using metallic punches. In the side holes, glass needles were inserted before the hydrogel was poured in.

Preparation of the Cubic Hydrogel for the Quantification of Bacterial Leakage. Activated methacrylated dextran solution was poured into a PDMS mold with a pin placed through the central hole from one side to the center of the cubic mold. Removal of the pin after the solidification of the pregel solution resulted in an empty chamber within the hydrogel for loading bacteria. The casted methacrylated dextran was kept frozen at −20 °C overnight, and later thawed at room temperature, washed with 20 mL of Milli-Q, and allowed to swell for 5 min. Next, the gel was washed with 20 mL of 0.05 M NaOH followed by 20 mL of Milli-Q and stored at 4 °C in a Falcon tube with fresh Milli-Q.

Hydrogel Disk Preparation. Activated methacrylated dextran was casted in a mold used for fabrication of acrylamide-based gels for SDS-PAGE (Mini PAGE mold, Bio-Rad). This resulted in the fabrication of a 0.75 mm-thick homogeneous sheet. After washing, the hydrogel disks were formed by punching off the sheet with a 8 mm hole puncher, and stored in fresh Milli-Q at room temperature.

Cloning. Cloning procedures were performed following the Gibson assembly method. Reagents and competent DH5α cells used for cloning were from the NEBuilder HiFi DNA Cloning Kit (Product ESS205 New England Biolabs). Q5 High-Fidelity 2X Master Mix was used for long PCRs (Product M0492S New England Biolabs 240 County Road Ipswich, MA 01938–2732). The correct gene products were confirmed by DNA sequencing (BaseClear BV. Sylviusweg 74 2333 BE Leiden, The Netherlands, Tables S1 and S2).

Bacterial Culturing. Unless otherwise noted, all bacteria were grown at 37 °C. Cultures were grown overnight in LB supplemented with selective antibiotics from single colonies of freshly transformed cells. Overnight cultures were further diluted 100-fold in fresh LB supplemented with selective antibiotics. After 3 h incubation, cells were chemically induced (0 or 5 mM arabinose, 0 or 0.5 mM IPTG) (OD₆₀₀ nm = 0.8) and further grown for 3 h. Note timing is important for ensuring optimum protein expression levels and therefore functionality of the system.

Bacterial Leakage Experiment with the Cubic Hydrogels (Liquid LB Medium). Cubic hydrogels were soaked in LB medium supplemented with 30 µg/mL chloramphenicol, 100 µg/mL kanamycin, 5 mM CaCl₂, and 0.5 mM IPTG for 1 h and transferred to an empty sterile Petri-dish by tweezers with the opening of the bacteria-loading chamber facing upward. Excess LB was pipetted out of the central chamber. Engineered E. coli (7 µL, OD₆₀₀ nm is approximately 1) were pipetted into the central chamber of the cubic hydrogel placed in a sterile Petri-dish. Next, 25 mL of medium was added to the plate gradually to roughly 80% (8 mm) of the height of the cubic hydrogel incubated at room temperature. The medium was collected at two different time points after the bacterial loading (10 min and 24 h) and plated (100 μL) for the quantification of bacterial leakage by CFU counting. The LB medium and Petri-dish were refreshed after each sample collection to prevent the overgrowth of bacteria.

Experiments for Assessing Bacterial Retention to the Hydrogels. Approximately 100 µL of activated methacrylated dextran solution was poured into individual wells of 96-well plate to make hydrogel cylinders. To bind bacteria to the dextran-based hydrogel, each hydrogel cylinder was incubated with 5 mL of MPA⁺ or MPA⁻ (OD ≈ 1) in the presence of 0.5 mM IPTG and 2 mM CaCl₂ in a shaking incubator overnight (37 °C, 250 rpm). The hydrogels were then
transferred to a fresh culture tube containing 5 mL of a sterile buffer (10 mM Tris-HCl, 150 mM NaCl, and 2 mM CaCl₂) with antibiotics (for Mpa⁺, chloramphenicol only; for Mpa⁻, both kanamycin and chloramphenicol). The hydrogels were washed in the buffer with shaking (200 rpm, 20 °C) for 1 h. The hydrogels were then transferred to fresh culture tubes and homogenized with a glass rod before resuspending in 100 μL of the wash buffer. Serial dilutions of the samples were plated onto agar plates with appropriate antibiotics, and CFUs were counted the next day for quantification of bacteria bound to the hydrogel.

**Scanning Electron Microscopy.** Dextran gels seeded with cells were first fixed in 4% formaldehyde for 20 min, and further kept in Milli-Q water. Samples were further sliced using a scalpel to show the cross-section of the bacterial loading chamber and sputter coated with a 9 nm-gold layer using an EMITECH K575X Peltier-cooled SOP Turbo sputter coater Dual. The gels were imaged at 5 kV in a FEI Quanta 3D field-emission SEM, equipped with an Everhart-Thornley secondary electron detector.

**Bacterial Leakage Experiment with the Hydrogel Disks (LB-Agar Medium).** Prior to use, dextran-based hydrogel disks were soaked in LB medium supplemented with 30 μg/mL chloramphenicol, 100 μg/mL kanamycin, and 5 mM CaCl₂. Next, 100 μL of a solution containing chemical inducers (0 or 5 mM arabinose, 0 or 0.5 mM IPTG) were spread on LB-agar plates supplemented with antibiotics and CaCl₂. The surface of the LB-agar substrate was allowed to dry by incubating the plates at 37 °C for 30 min. Hydrogel disks were set on the agar-LB surface and quickly dabbed dry using clean, dust-free, paper tissue, and further incubated for 10 min at 37 °C. A drop (1 μL) of engineered *E. coli* culture was placed in the center of hydrogel surface on the LB agar medium within Petri dishes. The migration of the engineered *E. coli* was tracked by imaging for 4 days. Pictures were analyzed using ImageJ/Fiji.51

**Antibacterial Activity Tests (Modified Kirby-Bauer Test).** Master mix solutions (100 μL) containing various chemical inducers (0 or 5 mM arabinose, 0 or 0.5 mM IPTG) were spread on LB-agar plates supplemented with antibiotics and CaCl₂. The surface of the LB-agar substrate was allowed to dry by incubating the plates at 37 °C for 30 min. Hydrogel disks were set on the agar-LB surface and quickly dabbed dry using clean, dust-free, paper tissue, and further incubated for 10 min at 37 °C. A drop (1 μL) of engineered *E. coli* culture was placed in the center of hydrogel surface on the LB agar medium within Petri dishes. The migration of the engineered *E. coli* was tracked by imaging for 4 days. Pictures were analyzed using ImageJ/Fiji.51

**Cell-Free Medium Preparation.** Approximately 3 h after lysostaphin induction with 5 mM arabinose, 100 mL of engineered *E. coli* cell culture was spun down by centrifugation (4000 rpm) for 15 min at 4 °C. The cell-free-medium was then harvested after the removal of residual cells by filtering (0.2 μm cutoff). To remove the antibiotics present in the growth medium, the cell-free-medium was buffer-exchanged into buffer A and concentrated approximately 30-fold using Millipore spin cassettes (molecular weight cutoff: 10-kDa). The cell-free medium was further tested by applying a drop of 5 μL onto the surface of the presterilized plates (see antibacterial activity tests).

**Living Hydrogel with anti-MRSA Activity.** Dextran-based hydrogel disks were briefly dried using a sterile cotton swab and then placed on the Mueller-Hinton agar plates streaked with methicillin-resistant *Staphylococcus aureus*. Prior to their infusion into the hydrogel disks, the engineered *E. coli* cells were washed to 2 times in LB to ensure complete removal of antibiotics (growth medium). Next, a drop (1 μL) of the induced *E. coli* cells was placed at the center of the dextran-based hydrogel disk surface. Plates were further incubated at 37 °C for up to 4 days, and the effects of bacteriocin-producing living hydrogels were visualized by imaging with a DSLR camera (Canon 600D, 50 mm lens). Pictures were analyzed using ImageJ.

## ASSOCIATED CONTENT

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.9b00404.

Quantification of unreacted monomers released from the hydrogel; characterization of dextran-based hydrogel microstructure; fabrication of cubic-dextran-based hydrogel; quantification of bacterial retention to the dextran-based hydrogel (PDF)

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