Red/Far-Red Light Switchable Cargo Attachment and Release in Bacteria-Driven Microswimmers

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In bacteria-driven microswimmers, i.e., bacteriabots, artificial cargos are attached to flagellated chemotactic bacteria for active delivery with potential applications in biomedical technology. Controlling when and where bacteria bind and release their cargo is a critical step for bacteriobot fabrication and efficient cargo delivery/deposition at the target site. Toward this goal, photoregulating the cargo integration and release in bacteria using red and far-red light, which are noninvasive stimuli with good tissue penetration and provide high spatiotemporal control, is proposed. In the bacteriobot design, the surfaces of *Escherichia coli* and microsized model cargo particles with the proteins PhyB and PIF6, which bind to each other under red light and dissociate from each other under far-red light are functionalized. Consequently, the engineered bacteria adhere and transport the model cargo under red light and release it on-demand upon far-red light illumination due to the photoswitchable PhyB–PIF6 protein interaction. Overall, the proof-of-concept for red/far-red light switchable bacteriabots, which opens new possibilities in the photoregulation in biohybrid systems for bioengineering, targeted drug delivery, and lab-on-a-chip devices, is demonstrated.

Biohybrid microrobots combine cells and synthetic cargos (mainly micro- and nanoparticles) for potential applications in biomedical technology including diagnostics and targeted delivery of sensitive materials, such as imaging agents, genes, and drugs. In this context, cells offer unique characteristics that make them well-suited as delivery agents, such as self-propulsion, environmental sensing abilities, production of biomolecules on-site, and taxis. Among different microorganisms that have been employed to this end, biohybrid microrobots with bacterial cells, i.e., bacteriabots, are particularly of interest due to their viability in diverse environments, robust motility, efficient conversion of chemical energy into mechanical energy, and straightforward genetic engineering. Most importantly, their ability to detect and follow gradients of diverse external stimuli (i.e., pH, oxygen, glucose, and temperature) provides a unique opportunity for controlling their taxis behavior. This feature has allowed designing bacteria to follow specific cues for active delivery of cargo based on chemotaxis, phototaxis, pH-taxis, thermotaxis, and magnetotaxis. The efficient integration of the cargo onto the bacteria and the controlled release of the cargo from the bacteria are critical factors for successful application of bacteriabots. To attach bacteria to the surface of cargo, different types of unspecific and specific interactions (electrostatic interactions, hydrophobic interactions, covalent attachment, streptavidin-biotin, and antibody-antigen) have been used. Yet, the controlled on-site release and active delivery of the cargo, which improve the bioavailability of the administration have only been demonstrated in a few studies. In these examples, the cargo has been released in response to external stimuli, such as chemicals, pH, and UV-light. Controlling bacterial attachment to and release from a synthetic surface with visible light instead of UV light would be especially attractive since it would offer better biocompatibility while preserving the bioorthogonal and high spatiotemporal control. In particular, for in vivo applications, photoregulation of bacteria-cargo interactions with red/near-infrared light (650–1350 nm) would be desirable due to its good tissue penetration.

Here, we report a new approach to photoregulate the interaction between bacteria and their cargo in bacteriabots using red/far-red light, which would offer good biocompatibility and overcome the above-mentioned limitations. In the design, we envisioned that the bacteria attach to the cargo upon red light illumination, transport it to the target site and release it on demand upon far-red light illumination. To photoregulate the cargo integration in bacteriabots, we employed the photoswitchable protein phytochrome B (PhyB, amino acids 1–651) from Arabidopsis thaliana, which under red light (660 nm) binds to the protein phytochrome interaction factor 6 (PIF6, amino acids 1–100). The red light-triggered binding of...
PhyB/PIF6 is reversible under far-red light (740 nm) within seconds (Figure 1).\textsuperscript{[31]} We thus engineered Escherichia coli (E. coli) bacteria to display PhyB on their surface, such that the bacteria could bind to PIF6 decorated cargo under red light illumination and release it under far-red light illumination. This design offers reversible and dynamic control over cargo attachment and release in bacteria and improves the spatiotemporal control of biohybrid systems with future possible applications in engineering, targeted drug delivery, and lab-on-a-chip devices.

To realize the envisioned design, we used the most commonly studied model organism for bacteriobots, E. coli, due to its well-characterized taxis abilities, easy genetic manipulation, established cell surface display systems, and wide use in bioengineering for therapeutic applications. To display the red light-switchable protein, PhyB, on the surface of E. coli, we utilized a biotinylated Ag43 (Ag43-biotin) protein expressed on the cell surface,\textsuperscript{[30]} which allowed linking recombinantly expressed and purified biotinylated PhyB through a streptavidin linker to the E. coli surface (Figure S1, Supporting Information). We chose this strategy for the surface functionalization of the bacteria with PhyB since the direct expression and display of large proteins, such as PhyB (651 aa) on bacteria surfaces is challenging. On the other side, the complementary interaction partner PIF6 was linked to Ni\textsuperscript{2+}-NTA functionalized materials as a PIF6-GFP-His6-tag, relying on the specific Ni\textsuperscript{2+}-NTA binding to His-tags.

As the first step of a red/far-red light controlled bacteriobot fabrication, we tested whether the photoswitchable PhyB/PIF6 interaction could be used to control the adhesion of bacteria to synthetic surfaces (Figure 2a). For this purpose, we initially immobilized PIF6 onto glass substrates with a poly(ethylene glycol) (PEG) coating and Ni\textsuperscript{2+}-NTA terminal groups. This PEG coating provided an inert background by preventing unspecific binding of proteins and bacteria and allowed for the specific immobilization of His-tagged PIF6 onto the glass substrate. Then, PhyB functionalized bacteria, which were fluorescently labeled for visualization, were incubated on these surfaces for 1 h either under far-red or red light (Figure 2b, i,ii). These PhyB displaying bacteria were only able to adhere to the PIF6 functionalized substrates under red light illumination, while no bacteria were observed on the substrate under far-red light. Also, the quantification of the bacteria that adhered to the substrate showed that the bacteria adhered significantly under red light and that the number of bacteria under far-red light and substrates that were not functionalized with PIF6 was insignificant (Figure 2c). Taken together, these results show that the PhyB immobilized onto the bacteria is accessible and active and that the bacteria adhesion is due to the highly specific red light-dependent PhyB–PIF6 binding.

Since the triggered reversion of the bacteria adhesions is the key point in releasing the cargo from bacteriobots, we investigated if and how fast the PhyB–PIF6 mediated interactions between bacteria and synthetic surfaces could be switched off under far-red light. When PhyB functionalized bacteria, which had adhered onto PIF6 functionalized glass substrates described above for 1 h under red light, were placed under far-red light for 30 min, all bacteria detached from the substrate (Figure 2b, iii). The time-dependent analysis of the detachment was showed that even after 1 min of far-red illumination 98% of the bacteria detached (Figure 2d). To verify that the observed differences in bacterial attachment were not a result of phototoxicity and to demonstrate the high biocompatibility of red/far-red light, we checked the viability of the bacteria and mammalian cells (MDA-MB-231 cells) under different light illumination (Figure S2, Supporting Information). This analysis showed that after 1 h illumination with different light sources used in this study there was no loss in viability compared to untreated cells. Overall, this demonstrates that the red/far-red light switchable PhyB/PIF6 interactions can be used to reversibly and noninvasively photocontrol the attachment and detachment of PhyB functionalized E. coli to PIF6 functionalized materials.

Once it was established that the binding of PhyB functionalized bacteria to PIF6 functionalized materials could be reversibly turned on and off using red and far-red light, we sought to control bacteria-cargo interactions using the PhyB–PIF6 binding. This would allow generating bacteriobots under red light using PhyB functionalized bacteria and PIF6 functionalized cargos and release the cargo at the desired location upon far-red light illumination (Figure 1). As a model for synthetic cargo, we used 2 µm Ni\textsuperscript{2+}-NTA functionalized magnetic polystyrene (PS) particles, which were functionalized with His6-tagged PIF6. In initial tests, E. coli, which were functionalized with PhyB and expressing GFP for detection, were incubated with these PIF6 functionalized magnetic particles under different illumination conditions. Subsequently, the bacteria interacting with the magnetic particles were separated using a magnet and quantified (Figure 3a). After 10 min of red light and far-red light illumination, the number of bacteria that adhered on the
particles under red light was substantially higher than under far-red light. Moreover, under red light illumination, these PhyB displaying bacteria (labeled with GFP) and fluorescently labeled PIF6 functionalized 2 µm PS particles form direct contacts and assemble into bacteriabots as observed with fluorescence microscopy (Figure 3b). To demonstrate the reversibility of this attachment, samples incubated for 10 min under red light illumination were subsequently either illuminated with far-red light or kept in the dark for 10 min. The number of bacteria adhering on the particles after the far-red light treatment was comparable to the sample which was only illuminated with far-red light. The adhered bacteria detach from the substrate upon far-red light illumination. Scale bars are 50 µm. c) Quantification of PhyB functionalized bacteria on PIF6 functionalized substrates under red light, under far-red light, and on PEG-coated substrates without PIF6. Error bars are the standard error from 15 images. d) Detachment kinetics under far-red light of PhyB functionalized bacteria from PIF6 functionalized substrates after 1 h red light illumination. Error bars are the standard error from 15 images of three biological replicates.

Figure 2. a) PhyB functionalized E. coli adhere on PIF6 functionalized substrates under red light and detach from the substrate under far-red light, due to the red light-dependent PhyB–PIF6 interaction and its reversion under far-red light. b) Fluorescence microscopy images of GFP expressing and PhyB functionalized E. coli functionalized on PIF6 functionalized PEG-coated glass substrates. i) 1 h far-red light. Bacteria did not adhere to the substrate. ii) 1 h red light. Bacteria adhere to the substrate. iii) 1 h red light followed by 30 min far-red light. The adhered bacteria detach from the substrate upon far-red light illumination. Scale bars are 50 µm. c) Quantification of PhyB functionalized bacteria on PIF6 functionalized substrates under red light, under far-red light, and on PEG-coated substrates without PIF6. Error bars are the standard error from 15 images. d) Detachment kinetics under far-red light of PhyB functionalized bacteria from PIF6 functionalized substrates after 1 h red light illumination. Error bars are the standard error from 15 images of three biological replicates.

Binding kinetics of bacteria to the cargo is an important aspect for bacteriabot fabrication and understanding the temporal modulation the PhyB–PIF6 interaction offers. At this point, we also investigated the contribution of unspecific interactions in the formation of bacteriabots. Therefore, we incubated bacteria with and without PhyB functionalization with PIF6 functionalized PS particles for up to 2 h under red light illumination (Figure 3c). Within 10 min of red light illumination, attachment of PhyB functionalized bacteria was twofold higher compared to the nonfunctionalized bacteria (blank). As the incubation time under red light increased, the number of PhyB functionalized bacteria that attach to the PIF6 beads increased rapidly and reached a plateau around 60 min. On the contrary, the unspecific binding between particles and bacteria did not increase significantly with time and was similar to the attachment of PhyB displaying bacteria under far-red light after 2 h incubation. Although the PhyB–PIF6 interaction is highly specific as also demonstrated with the PEG-coated substrates, unspecific interactions between E. coli and the PS particles
contribute to the binding of bacteria to the model cargo. Therefore, the contribution of unspecific interactions needs to be considered in the design of photoswitchable bacteriabots.

As the complementary process, the reversion of the bacteria particle interaction was studied to gain insight into how quick cargo can be released from the bacteriabots. For this purpose, bacteriabots were first assembled under red light for 10 min before illuminating them with far-red light or placing them in the dark. We observed that within 5 min of far-red light illumination most of the bacteria disassociate from the particles and the number of bacteria attached to the particles was comparable to the unfunctionalized control bacteria (Figure 3d). On the other hand, the bacteriabots remained intact when placed in the dark over 30 min. The lack of reversibility in the dark is actually an advantage as it shows that once the bacteriabots are assembled under red light, illumination can be stopped and the bacteriabots will remain intact until illuminated with far-red light. It should be noted that if PhyB functionalized bacteria and PIF6 functionalized particles were incubated under red light for 30 min rather than 10 min, the attached cargo could not be released even after 30 min far-red light illumination. This lack of reversibility after longer bacteria-bead contact could be due to secondary nonspecific interactions that form when bacteria are in close proximity to the PS particles. In fact, the binding of PhyB and PIF6 reverses within seconds at the molecular level. This fast reversion was also mirrored in the complete detachment of bacteria from PIF6 functionalized PEG-coated substrates within 1 min. To achieve faster cargo drop in future bacteriabots, the cargo should be coated with PEG like molecules that prevent undesired secondary interactions. In this sense, the quick and specific reversion of the PhyB–PIF6 interactions with far-red light is critical for the cargo release.

As a final step, we demonstrated that the red light-triggered assembly of the bacteriabots and far-red light-triggered detachments of the cargo from the bacteria can be used to control cargo transport in bacteriabots. For this purpose, we evaluated the movement of cargo particles (fluorescently labeled PIF6 functionalized 2 µm PS particles) transported by bacteriabots moving in a chemotactic gradient (Figure 4). This analysis showed that particles transported by bacteriabots assembled for 10 min under red light had a mean speed of 8.44 µm s⁻¹. On the other hand, when the cargo was released from these bacteriabots by illuminating them for 10 min with far-red light, most particles remained still and the mean speed dropped to 4.22 µm s⁻¹. Likewise, for the tracked particles the accumulated distance and the Euclidean distance as well as the forward migration index (FMI) in the chemotactic gradient, showing directional movement, dropped after far-red light illumination (Figure S3, Supporting Information). Furthermore, the mean fluorescence microscopy image of the GFP labeled PhyB functionalized E. coli (shown in green) interacting with a PIF6 functionalized 2 µm PS particle (shown in red). ii) Optical microscopy image in bright field. Scale bar is 5 µm. c) Attachment kinetics of PhyB functionalized and unfunctionalized E. coli to PIF6 functionalized PS particles under red light. d) Reversion kinetics of PhyB functionalized E. coli and PIF6 functionalized PS particles under far-red light and in the dark after 10 min red light illumination. Error bars show standard error of the mean from three biological replicates each done in three technical replicates.
speed of unfunctionalized PS particles, used as a negative control to account for random movement, was only 3.29 μm s⁻¹. Also, the FMI was similar for the negative control and reversed sample, further showing that the cargo was released and is no longer transported by the bacteria.

In summary, we developed a red/far-red light switchable specific bacterial adhesions to synthetic materials based on the photoswitchable PhyB–PIF6 interactions. These photoswitchable interactions were employed to photoregulate the integration of cargo into bacteriabot using benign visible light and providing unprecedented control over the cargo-bacteria interphase in biohybrid microrobots. The assembly of the bacteriabot under red light, the stability of the once formed bacteriabot in the dark and the fast cargo release within 10 min under far-red light at the desired location are ideal for later applications in vivo due to the good tissue penetration of red/far-red light. As a proof-of-concept, our results demonstrate the possibility of a dynamic and effective cargo integration and release in bacteriabots with red and far-red light, respectively. This study paves the way toward improving the control of biohybrid systems in bioengineering, targeted drug delivery, and lab-on-a-chip devices.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
bacteria adhesion, bacteriabots, photoswitchability, PhyB/PIF6, reversibility

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