Bacterial Recognition

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Bacterial Imprinting at Pickering Emulsion Interfaces**

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Abstract: *The tendency of bacteria to assemble at oil–water interfaces can be utilized to create microbial recognition sites on the surface of polymer beads. In this work, two different groups of bacteria were first treated with acryloyl-functionalized chitosan and then used to stabilize an oil-in-water emulsion composed of cross-linking monomers that were dispersed in aqueous buffer. Polymerization of the oil phase followed by removal of the bacterial template resulted in well-defined polymer beads bearing bacterial imprints. Chemical passivation of chitosan and cell displacement assays indicate that the bacterial recognition on the polymer beads was dependent on the nature of the pre-polymer and the target bacteria. The functional materials for microbial recognition show great potential for constructing cell–cell communication networks, biosensors, and new platforms for testing antibiotic drugs.*

Research at the intersection of microbiology and nano/microfabrication is an enormous challenge.[1] Within this context, the controlled self-assembly of microorganisms is a subject of substantial interest in many biomedical and bacteriological studies (e.g., cell–cell communications and cell–surface interactions).[1b] Thus far, controlled bacterial assembly has been achieved mainly by soft lithography patterning and through cell-wall engineering.[2] These strategies exploit the chemical interactions between bacteria and surfaces to control microbial adhesion. The existing methods to pattern bacteria-binding surfaces are incapable of offering high selectivity; therefore, it is necessary to develop new synthetic strategies that can provide specific bacterial recognition surfaces.

One possibility to obtain selective bacterial recognition surfaces entails the use of the molecular imprinting technique.[3] Molecular imprinting allows the preparation of synthetic polymers (molecularly imprinted polymers, MIPs) with specific binding sites for pre-determined target molecules.[4] Because of their high specificity, MIPs have found many practical applications spanning affinity separation, catalysis, and chemical sensing.[5] However, when large templates (e.g., proteins, viruses, and bacteria) are imprinted, some non-trivial problems are encountered.[5c,d] First, as bacteria are living organisms, they are not rigid, and it is therefore difficult to generate well-defined cavities during an imprinting reaction. Second, traditional MIPs are highly cross-linked, making it difficult for bacteria to reach the binding sites, which are buried in the interior of the MIPs. To overcome these difficulties, several new approaches, which entail the use of the "stamping method",[6] sol–gel chemistry,[7] colloidal imprints,[8] or lithographic processes,[9] have been widely used to create bacterial imprints on the polymer matrix.

Herein, we developed a novel method to synthesize bacterial recognition polymers by exploiting the capability of bacteria to self-assemble at an oil–water interface. By selecting pre-polymers (such as carbohydrates) that show a high affinity for the target bacteria, we demonstrate that bacterial recognition polymers can be prepared from emulsions that are stabilized by bacteria-based pre-polymer networks. Particle-stabilized emulsions are also known as Pickering emulsions.[10] The basic principle of droplet stabilization is the partitioning of solid particles between two immiscible liquids.[10a] Since they were first reported, Pickering emulsions have been exploited in various applications, such as oil recovery, cosmetic preparations, waste water treatment, and fabrication of functional materials with complex architectures.[10b] For example, using Pickering emulsion polymerization, MIPs that are capable of recognizing small organic molecules and proteins have been synthesized in our laboratory.[11] Aside from amphiphilic solid particles, living microorganisms have also been used as particle stabilizers to prepare Pickering emulsions.[12] In this context, the self-assembly of a bacteria–chitosan network at the oil–water interface was found to be sufficient to stabilize the Pickering emulsion.

Our synthetic approach for preparing a bacteria-imprinted polymer (BIP) by Pickering emulsion polymerization is shown in Scheme 1. First, negatively charged bacteria were assembled with a positively charged vinyl-containing pre-polymer, and the obtained bacteria–pre-polymer complex was then used as the particle stabilizer to construct a stable emulsion of a cross-linking monomer (the oil phase) in water. The oil phase was polymerized by means of free radical initiation, which also caused the pre-polymer to be covalently fixed to the core of the polymer beads. In the last step, the bacterial template was removed from the polymer beads, leaving behind bacteria-imprinted sites on the bead surface. The advantage of this method resides in its general applicability, as a large variety of pre-polymers can be

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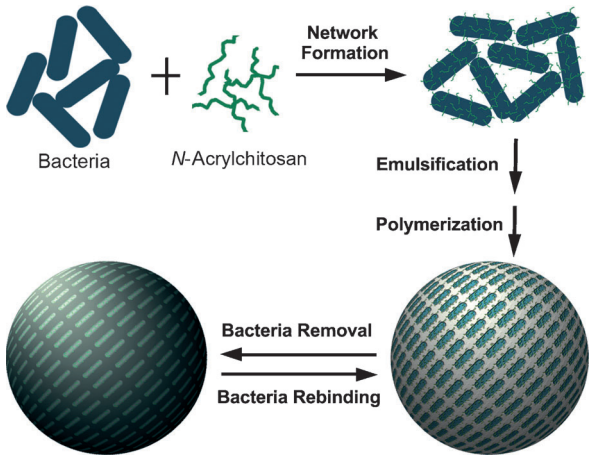

Scheme 1. Interfacial bacteria imprinting by Pickering emulsion polymerization.

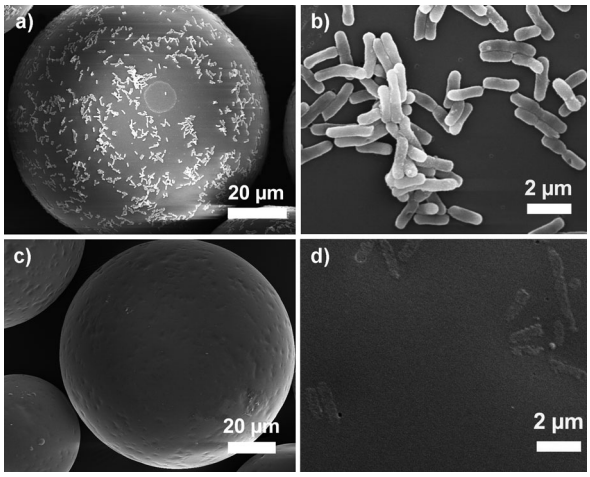

Figure 1. a–d) SEM images of polymer beads imprinted with bacteria before (a, b) and after (c, d) removal of the *E. coli* template.

selected based on their specific molecular interactions with the target bacteria.

Here, the vinyl-containing pre-polymer, *N*-acrylchitosan (NAC) was synthesized by reacting acryloyl chloride with the amino groups of the glucosamine units in chitosan (Supporting Information, Figure S1). The fluorescence spectrum shown in Figure S5 indicates that approximately 16% of the amino groups on chitosan were acrylated. A low degree of acrylation was used deliberately to make sure that the obtained NAC has enough free amino groups to bind the bacteria through electrostatic interactions.[12a] The pendant acryloyl groups in the modified chitosan can still allow the co-polymerization of NAC with the cross-linkers in the oil phase.

During the preparation of the Pickering emulsion, the oil phase was composed of cross-linkers and the initiator. After mixing the oil phase with an aqueous suspension of the bacteria–NAC complex, a stable emulsion was obtained by shaking the mixture vigorously (Figure S6c). It should be noted that no stable Pickering emulsion could be obtained when the bacteria or NAC were used on their own (Figure S6), indicating that the bacteria–NAC complex was essential for stabilizing the Pickering emulsion (Figure 1). This result is in agreement with the literature,[12a] as the bacteria–chitosan network was suggested to irreversibly cover the oil–water interface in the Pickering emulsion.

The stable Pickering emulsion was polymerized at room temperature. After removal of the bacteria templates by solvent extraction, BIP beads were obtained. The BIP beads were named *E*-BIP and *M*-BIP when rod-shaped *Escherichia coli* (*E. coli*) cells and spherical *Micrococcus luteus* (*M. luteus*) cells were used as the bacterial templates, respectively. The fluorescence microscopy images in Figure S9 indicate that the particle size of the BIP beads is 110 ± 17 μm. The surface morphology of the BIP beads before and after bacterial removal was studied by scanning electron microscopy (SEM). Before solvent extraction, the *E*-BIP beads that were prepared in phosphate-buffered saline (PBS) containing the template bacteria ($OD_{600} = 2$, ca. 5×10^8 cfu mL^{-1}) had clearly visible *E. coli* cells on their surface (Figure 1a, b and Figure S7). After the bacteria had been removed, tailor-made cavities were left on the surface of the *E*-BIP beads, which were easily accessible for bacteria binding (Figure 1c, d). The recognition performance of the bacteria on the BIP beads was first studied by using the following bacterial species as representatives: *E. coli*, *Lactobacillus sp.* (*LB790*), *M. luteus*, and *Enterococcus faecium* (*E. faecium*; Table 1).[13] Figure 2a and 2b show the adsorption of *E. coli* and *M. luteus* bacteria by *E*-BIP and *M*-BIP beads. Both *E*-BIP and *M*-BIP beads showed preferential binding of the corresponding template cells, indicating that BIP beads possessed high selectivity between rod-shaped and spherical bacteria. Interestingly, this selectivity between the rod-shaped and spherical bacteria was stronger than the selectivity between cells within the same group (see Figure 2c, d). The selective binding of bacteria suggests that the present bacterial imprinting method at Pickering emulsion interfaces is an effective way to produce specific bacterial recognition surfaces. However, the microbial imprinting has not reached the level of precision that can be achieved by imprinting small molecules.

To confirm the importance of the pre-polymers for bacterial binding, we passivated the amino groups on the

Table 1: Displacement (*DP*) of bound mCherry–*E. coli* from *E*-BIP beads with different competing bacteria.

Competitor	*E. coli*	*B. sp.*	*LB790*	*E. faecium*	*M. luteus*
ζ Potential[a] [mV]	−20[12a]	−15[13b]	−5[13c]	−30[13d]	−10[13e]
Shape	rod	rod	rod	sphere	sphere
Size [μm]	ϕ (0.4–0.8) × 2.0	ϕ (0.6–0.9) × 1.5–4.0	ϕ (0.7–1.1) × (2.0–4.2)	ca. ϕ 0.8	ca. ϕ 0.8
DP[b] [%]	63.0 ± 4.0	38.3 ± 8.5	37.1 ± 3.9	30.4 ± 5.4	7.7 ± 0.9

[a] At approximately pH 7. [b] The displacement (*DP*) of mCherry–*E. coli* was calculated as $DP\,[\%] = [(\text{bound}_0 - \text{bound})/\text{bound}_0] \times 100$, where bound_0 and bound are the amount of mCherry–*E. coli* cells ($OD_{600} = 0.03$) bound by *E*-BIP beads in the absence and presence of the competing bacteria ($OD_{600} = 0.24$), respectively. Conditions: Beads (50 mg) in PBS buffer (pH 7.2; 1 mL), incubated at 4 °C for 3 hours.

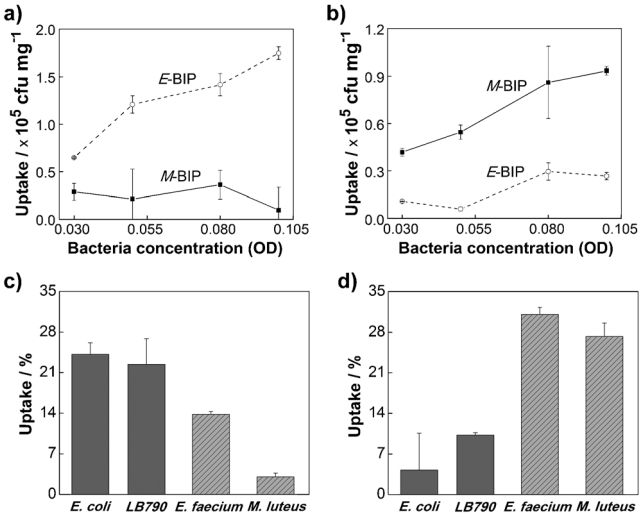

Figure 2. a, b) Uptake of *E. coli* (a) and *M. luteus* (b) cells with different concentrations by *E*-BIP and *M*-BIP beads. c, d) Uptake of different cells ($OD_{600} = 0.05$) by *E*-BIP (c) and *M*-BIP (d) beads. Conditions: Polymer beads (50 mg) in PBS buffer (pH 7.2; 2 mL), incubated with bacteria at 4 °C for 3 hours.

BIP beads by acylation reactions with three different acid anhydrides. The *E*-BIP beads that were treated with acetic anhydride, maleic anhydride, and trifluoroacetic anhydride were named RP-A, RP-M, and RP-T, respectively. The uptake of *E. coli* expressing green fluorescent protein (GFP–*E. coli*) by different polymers was then studied. In Figure 3a, the *E*-BIP beads displayed higher *E. coli* binding than the reference polymer beads, suggesting that the electrostatic interaction between the cationic NAC on the polymer beads and the negatively charged *E. coli* surface played an important role for bacterial binding. The negatively charged RP-M beads (bearing carboxyl groups on the surface) showed almost no binding of *E. coli* cells, indicating that the negatively charged surface effectively repelled the *E. coli* cells. Compared to the RP-M beads, the reference polymer beads that were covered by alkyl (RP-A) and fluoroalkyl groups (RP-T) showed higher *E. coli* binding, presumably because of hydrophobic interactions.

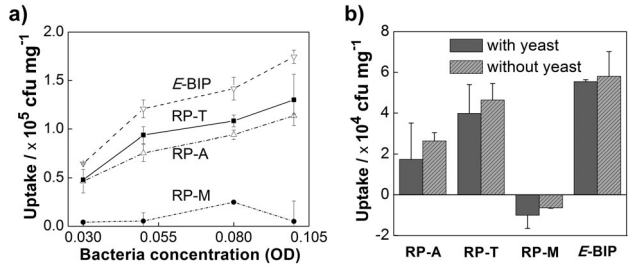

Figure 3. a) Uptake of GFP–*E. coli* cells measured at different initial concentrations. Conditions: Polymer beads (50 mg) in PBS buffer (pH 7.2; 2 mL), incubated with GFP–*E. coli* at 4 °C for 3 hours. b) Uptake of GFP–*E. coli* cells ($OD_{600} = 0.03$) in the presence of yeast cells ($OD_{600} = 0.24$). Conditions: Polymer beads (50 mg) in PBS buffer (pH 7.2; 1 mL), incubated at 4 °C for 3 hours.

The selectivity of bacteria binding on BIP beads was further investigated by measuring the uptake of GFP–*E. coli* cells in the presence of excess *Saccharomyces cerevisiae* yeast cells. As shown in Figure 3b, addition of the yeast cells had almost no influence on the uptake of the target *E. coli* cells by the different polymer beads. This result is due to the microbial size: The much larger yeast cells do not fit into the sites that were imprinted with *E. coli*. Despite the strong negative charge on the surface (with a ζ potential of -39.3 mV measured in water[13a]) and the larger size, the yeast cells were unable to compete with *E. coli* cells for the imprinted sites. Therefore, aside from the electrostatic interactions, the size of the bacteria also affects their binding.

To confirm the importance of chemical recognition and physical size for bacteria binding, competitive bacterial binding experiments were carried out. Here, *E. coli* expressing mCherry (mCherry–*E. coli*) cells were selected instead of GFP–*E. coli*, because mCherry–*E. coli* feature a longer emission wavelength (612 nm) and lower background interference. During the binding, an excess of various bacteria was added to a mixture of *E*-BIP beads and mCherry–*E. coli* ($OD_{600} = 0.05$) in PBS. Depending on the shape and the surface charge similarity, the added bacteria were expected to displace the mCherry–*E. coli* from the *E*-BIP beads to different degrees. Table 1 shows that mCherry–*E. coli* were most efficiently displaced by *E. coli* (without mCherry) > *LB790* ≈ *Bacillus sphaericus* (*B. sp.*) > *E. faecium* > *M. luteus*. This order of efficiency can be explained by comparing the electrostatic nature and the size of the mCherry–*E. coli* and the competing bacteria. For example, the *E. coli* cells (without mCherry) have the same shape and surface charge as mCherry–*E. coli*; therefore, *E. coli* cells efficiently displaced the mCherry–*E. coli* from the *E*-BIP beads. *M. luteus* cells, which are spherical bacteria with a negative charge surface, showed only a low effect on the binding of mCherry–*E. coli*. Interestingly, *LB790* (with a higher ζ potential and a larger size) and *B. sp.* (with a lower ζ potential and a smaller size) showed the same degree of displacement. Moreover, compared to *M. luteus*, *E. faecium* bacteria (which are similar in size to *M. luteus* but differ in the ζ potential) showed a higher degree of *E. coli* displacement, which agrees with the results presented in Figure 2c and d. These results confirm that the binding of bacteria by the BIP beads is affected by both the nature of the pre-polymers and the size of the target bacteria.

Figure 1a shows that *E. coli* cells self-assembled into aggregates during the Pickering emulsion polymerization, suggesting that the bacteria-imprinted surface may be used to deplete the microorganism more efficiently. To confirm this hypothesis, we incubated *E*-BIP and RP-M beads with a high concentration of *E. coli* in both PBS buffer and water, and carried out SEM analysis for the polymer beads. In PBS, a high density of *E. coli* was observed on the *E*-BIP beads (Figure 4a), whereas RP-M showed a much lower bacteria density on the surface (Figure S12d). In water, significant *E. coli* binding to the *E*-BIP surface was also observed (Figure 4b; see also Figure S13a, b). Again, no obvious self-assembly of cells could be observed on the reference polymer (Figure S12, S13). Based on these results, we suggest that the bacteria-imprinted sites on the *E*-BIP surface facilitate initial

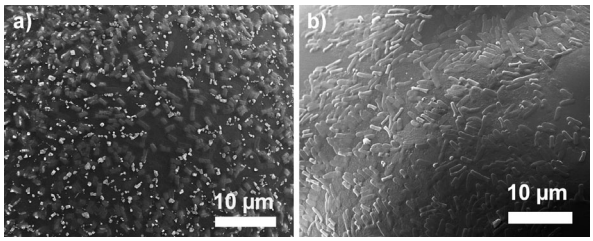

Figure 4. a, b) SEM images of bacteria self-assembly on *E*-BIP beads in PBS buffer (a) and in water (b). In (a), the white crystals are from the PBS buffer. Conditions: Polymer beads (5 mg) incubated in 1 mL of an *E. coli* suspension ($OD_{600} = 0.5$) at 4 °C for 24 hours.

E. coli binding, and the surface-bound *E. coli* could attract more bacterial cells through a self-assembly process. Obviously, the possibility of using imprinted surfaces to control cellular self-assembly can have practical applications, for example, in studying cell differentiation, preventing pathogen spreading, and tissue engineering.

In conclusion, using bacteria as particle stabilizers and at the same time as microbial templates, we prepared BIP beads using Pickering emulsion polymerization. During the imprinting, the pre-polymers that matched the target cell surface could be preferentially selected. This synthetic strategy is versatile and can be extended to a large variety of biological supramolecular systems as long as they can be surface-activated by complexation with suitable functional pre-polymers. The imprinting reaction is straightforward and can be scaled up. The binding experiments of the two groups of bacteria provide strong evidence that bacterial recognition on the BIP beads is dependent on the nature of the pre-polymers and the target bacteria. Interestingly, the bacteria-imprinted sites could facilitate the self-assembly of the target bacteria under overloading conditions. For biological applications, similar cellular imprinted surfaces may be utilized to control cell–cell communications and differentiation. The fine structure of the imprinted surface may also provide special microenvironments to improve cell viability, and thus lead to many new exciting possibilities in the life sciences.

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