Bacteria-Mediated Lithography of Polymer Surfaces

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Received January 16, 1996

The remarkable examples in nature of molecular recognition have inspired chemists to embark on the design and construction of synthetic receptors which can mimic biological systems in terms of selective interactions with ligands, structural reorganization, and even self-assembly into supramolecular architectures.[1−4] In practical terms, this involves the preparation of low molecular weight "building blocks" designed to interact either with a template or with each other via "complementary" surfaces.[5−19] Similarly, the assembly of a recognition site around a template molecule can be achieved within highly cross-linked polymeric matrices using molecular imprinting techniques[20−24] where the complementary functionality is introduced in the form of polymerizable monomers. However, all the work carried out within these rapidly developing areas so far has been concerned with relatively simple organic molecules. It would be of great interest to ascertain whether it is feasible to employ large macromolecular aggregates or even whole cells as templates to create complementary surfaces. In order to effect such a synthesis, it is necessary (i) to develop an approach enabling the precise reproduction of the size and shape of the template microorganism and (ii) to position appropriate functionality within the site while at the same time ensuring that the rest of the surface remains chemically inert. This Communication describes a novel bacteria-mediated lithographic procedure, where the cells in effect act as temporary protecting groups and structural templates, thus enabling us to achieve both objectives.

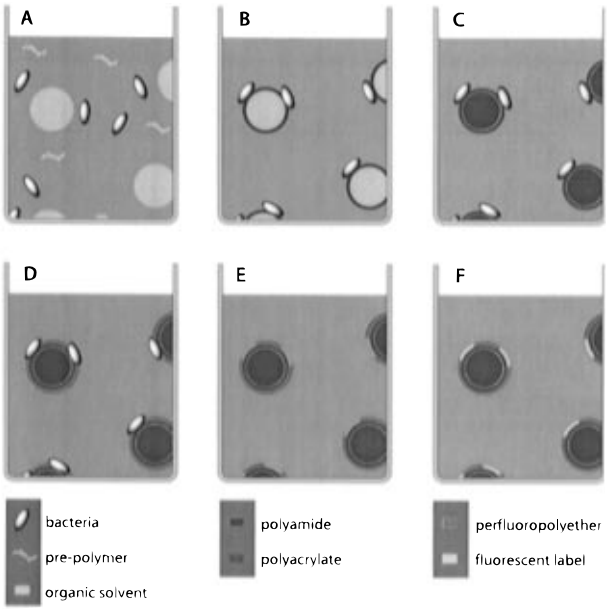

Figure 1. Schematic illustration of the bacterial lithographic process: (A) suspension of bacteria in an aqueous medium containing a dispersed organic phase, (B) formation of polyamide microcapsules and attachment of bacteria, (C) polymer beads after cross-linking of the microcapsule organic core, (D) modification of unprotected areas of the polymer surface with perfluoropolyether, (E) polymer beads after removal of bacteria to expose sites. (F) lithographic prints of bacteria after reaction with fluorescent lectin. A solution of 3(*N*-morpholino)-propanesulfonic acid (MOPS) buffer (pH 7.8, 0.6 N, 250 mL) was purged with nitrogen before addition of adipoyl chloride (1.2 mL) in a mixed organic phase containing dibutyl ether (14.4 mL), 1,6-hexanedioldiacrylate (14.4 mL), and azobis(isobutyronitrile) (300 mg). A suspension of bacteria (*L. monocytogenes* or *S. aureus*, 4×10^8 cfu·mL^{-1}), in MOPS buffer (50 mL), was then added, and stirring was continued for 3 min before dropwise addition of poly(allylamine) solution (0.12 N in 0.6 N MOPS, 75 mL) (A). The resultant microcapsules (B) with attached bacteria were irradiated (360 nm) with stirring for 12 h to generate solid beads (C) which were filtered, washed with water (3×100 mL) and methanol (3×100 mL), and air-dried. The beads (5.0 g) were then stirred in 1,1,2-trichlorotrifluoroethane (250 mL) as a solution of diisocyanato-terminated perfluoropolyether (FOMBLIN Z DISOC, 1.5 g) in 1,1,2- trichlorotrifluoroethane (20 mL) was added dropwise. Stirring was continued for 3 h (D) before addition of the suspension to methanol (250 mL). After filtration and methanol washing (5×100 mL), the beads were refluxed in 6 M HCl/methanol (150 mL) to remove the bacteria (E). "Development" of the exposed lithographic prints (F) was effected by stirring the beads (100 mg) in pH 4.75 sodium acetate buffer (5 mL, 50 mM, containing 5 mM $MnCl_2$, 5 mM $CaCl_2$, and ethanol (500 μL)) with fluorescein isothiocyanate-labeled Concanavalin A (1 mg, ∼0.0001 mmol) and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (5 mg, 0.025 mmol).

The protocol employs a range of chemistries, initially under mild conditions and neutral pH to ensure biocompatibility, as illustrated schematically in Figure 1. In the first step, we reacted a water soluble poly(amine), and a diacid chloride in a dispersed organic phase, in the presence of a stirred suspension of bacteria. The well-known tendency of microorganisms to partition at the interface between aqueous and organic layers, combined with the presence of the diacid chloride, capable of forming covalent bonds between nucleophilic groups in the bacterial cell walls and the poly(amine), ensured that the templates were positioned

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S0002-7863(96)00123-0 CCC: $12.00

and held at the loci of polycondensation. Thus, it was possible to effect the synthesis of a polyamide layer under and around the template bacteria, to yield microcapsules with cells in "sites" at the surface. This was followed by photoinitiated polymerization of a diacrylate, contained in the organic phase, to yield beads with a solid core. The resulting materials were treated with a diisocyanato-terminated perfluoropolyether,[25] to block any remaining amino groups on the exposed surfaces. The template bacteria, still in place, prevented access of the polymeric diisocyanate into the sites during this step. The microorganisms were then removed by acid hydrolysis, resulting in the formation of multilayered beads with spatially-defined, highly functional patches of size and shape corresponding exactly to those of the template bacteria. This whole process can be considered as the preparation of a "lithographic print" of the microorganisms.

The procedure was monitored using a combination of confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) (Figure 2). Plates a and b show the initial polyamide microcapsules, containing a liquid organic core, with surface-bound *Listeria monocytogenes* and *Staphylococcus aureus* as representative rod-shaped and coccoidal bacteria, respectively. The cells were labeled with the fluorescent dye ethidium bromide for this experiment. It is evident from the optical slices shown that the microorganisms were located on the outside of the polymer capsules, and the degree of surface coverage was easily controlled by variation of initial microorganism concentration and polymerization conditions. Photomicrographs of polymeric beads obtained after irradiation of the respective capsules are presented in plates c and d (Figure 2). Examination of the surface by SEM showed a certain degree of variation with regard to the position of the bacteria, with some cells almost completely buried in the outer layer of the polyamide and the majority only slightly embedded in the surface. After removal of the template microorganisms, the presence of deep indentations (100−200 nm) was readily apparent in SEM micrographs (Figure 2, plates e and f). These "prints", although infrequent compared to more shallow functionalized sites, exhibited the exact size and shape complementarity to the bacteria. However, to demonstrate the success of our lithographic procedure, it was necessary to "develop" the difference in chemical functionality between the now exposed sites and the perfluoropolymer-modified surfaces. The beads were therefore reacted with a fluorescently labeled lectin (FITC-Concanavalin A) via 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) mediated coupling of free amino groups on the unmodified polymer surfaces with carboxyl residues on the lectin. This enabled us to achieve a chemical amplification of the site functionality, where the lithographic image of the cells on the polymeric surface is enhanced and developed through the use of the fluorescent dye (plates g and h, Figure 2). Exactly the same procedure could be used for the introduction of a specific ligand (antibodies or lectins) for selective recognition of the template microorganism. Indeed, in preliminary experiments, some discrimination between bacteria was observed using the broad affinity lectin Concanavalin A.[26]

In conclusion, we have shown that it is possible to recreate the shape and size of whole cells employed as templates during multicomponent polymer synthesis. The resultant polymeric beads, exhibiting functionally anisotropic patches of dimensions defined by the template, can be further modified to adjust the chemistry in the sites and/or to introduce any further recognition elements required for a particular use.

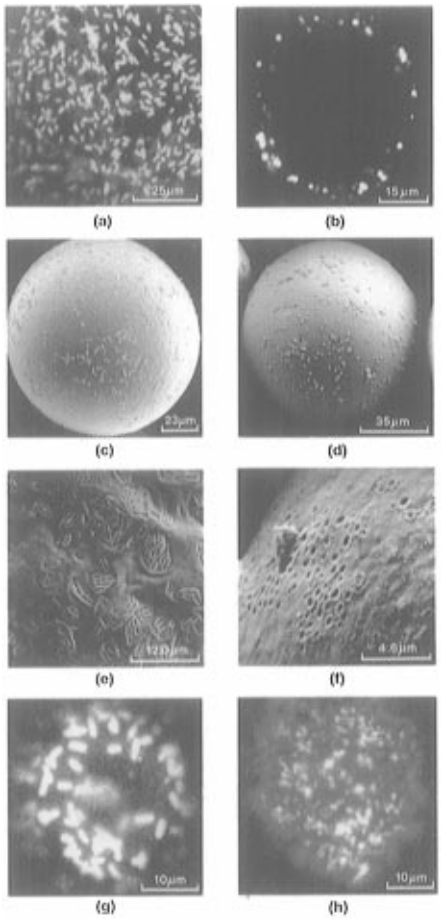

Figure 2. Micrographs of the bacteria-mediated lithographic process. Plates a and b: Confocal laser scanning micrographs (Zeiss LSM 1D) of ethidium bromide-stained *L. monocytogenes* and *S. aureus*, respectively, attached to polyamide microcapsules. (a) depicts the upper surface of a microcapsule with *L. monocytogenes* showing clearly in fluorescence, indicating the density of cell coverage. (b) is an optical slice through the middle of a polymer microcapsule: the fluorescent *S. aureus* cells delineate the outer surface of the polymer membrane. Plates c and d: Scanning electron micrographs (Hitachi S570) showing polymer beads after cross-linking of the diacrylate-containing organic core. The microorganisms can be seen partially embedded in the surface. The retention of the rod and coccoid shapes indicates that the gross physical morphology of the cells was unaffected by the polymerization process. Plates e and f: Scanning electron micrographs depicting the lithographic prints of the respective bacteria. The size and shape of the prints can be seen to correspond exactly to those of the microorganisms. In addition to the shape anisotropy, these sites were rendered distinct chemically by reaction with a diisocyanato-terminated perfluoropolyether, thus blocking the areas of the polymer surface not covered by the microorganisms. Following hydrolysis to remove the cells, the original functionality at the sites was exposed, allowing for further derivatization (development of the lithographic prints). Plates g and h show the "chemically-amplified" prints of the bacteria. After removal of the bacteria, the beads were reacted with a fluorescently labeled lectin, FITC-Concanavalin A. Confocal laser scanning microscopy depicts an optical section across the upper surface of the polymer beads, with the bright regions corresponding to areas reactive toward FITC-Concanavalin A. The anisotropic functionality of the surfaces can be seen to match exactly the dimensions of the bacteria in plates a and b and the sites in plates e and f, thus establishing the lithographic prints of the bacteria both topologically and chemically.

Acknowledgment. We thank MAFF for financial support.

JA960123C

(25) A perfluoropolyether was chosen for its combination of low adhesion, due to the fluorocarbon component, and hydrolytic stability.

(26) Assessment of binding was carried out by incubation of beads (50 mg) in MOPS buffer (5 mL, 10 mM, pH 7.0) with the appropriate microorganism (100 μL, 10^4 cfu·mL^{-1}). After rotation (2 h) samples of supernatant (100 μL) were removed for cell counts. Beads lithographed using *L. monocytogenes* showed better adsorption of this microorganism than those using *S. aureus* by a factor of 2, whereas the opposite was true for those "printed" with *Staphylococcus*: preferential binding of *Staphylococcus* over *Listeria* with ratios of up to 8:1 was observed in this case. Work is in progress to raise specific antibodies to enhance the binding properties of the lithographic prints.