Living Materials

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# Incorporation of Penicillin-Producing Fungi into Living Materials to Provide Chemically Active and Antibiotic-Releasing Surfaces**

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Medical devices and consumer-exposed surfaces with pre-loaded active ingredients and controlled release offer fascinating applications.[1] Their action, however, is time-limited, which is due to a non-replenishable stock of reactive compounds.[2] We directly implement the microorganisms synthesizing the reactive compounds into flexible polymer surfaces and thereby combine concepts from biotechnology with material science. These functional living materials make use of recently developed artificial biological niches,[3] where a nanoporous top layer constrains microorganism habitats in a 2D geometry. Using fungi and bacteria strains, we show how the defense mechanism of penicillin-producing fungi is maintained across nanoporous membranes, where it can be used to create surfaces that actively kill penicillin-susceptible bacteria. The concept introduced herein of biotechnological production on-site provides a route to complex and adaptive functional materials with sustained long-term release of specific compounds.

In the past decades, plenty of new and responsive materials have been developed. Compared to materials with a specific passive function, classical engineered materials have started to combine various properties. Such materials often have a pre-loaded function that is unleashed over time and converges to a minimum (for example silver-containing surfaces[4]). Recently developed smart materials are responsive to environmental stimuli and exhibit a desired property only when needed.[5] Stimuli can range from mechanical stress to temperature,[6] pH,[7] and magnetic[8] or electric fields.[9] Some types of smart materials are already common: Chemo-mechanical hybrid actuation systems enable pH-responsive motion of microstructures in liquids,[10] for example for artificial muscles[8,11] or chromogenic systems[12] for energy-saving windows. Stimuli responsive polymers provide controlled drug release[2a,13] or self-repairing slippery surfaces.[14] Biological or even ecological functions, however, are more challenging: Fighting bacterial contamination for example has become difficult as a result of resistance development after broad and unspecific release of antibiotics.[15] Smart materials with controlled release have a fixed supply of active ingredients, which usually depletes within weeks, requiring exchange or refill for prolonged function. Studies on antibiotic substance-releasing surfaces have recognized the need for prolonged activity as early as 1942.[16] An approach shown to increase the time of antimicrobial activity is the covalent linkage of antimicrobial substances to the surface.[17] In a further step, the living materials introduced herein produce and release active ingredients (Figure 1) continuously or upon bacterial contamination.[18] The present work targets a material that maintains long-term and sustained release of active compounds by incorporating the biotechnological production unit (that is, the living microorganism) inside a so-called living material.[3] In the test setup, we show activity against planktonic bacterial contamination without testing effectiveness against biofilms. *Penicillium chrysogenum* was chosen as one of the best-investigated fungi for the biotechnological production of penicillin, a key class of antibiotics. The fungus *Penicillium roqueforti* was used as a non-penicillin producing control organism. Suitable artificial 2D habitats for the fungi were created between a mechanical polymer support (100 micrometer polyacrylate layer) and a nanoporous top layer (10 μm thick, 400 nm pore size) and consisted of a modified agar and the organism itself (structural details are given in the Supporting Information). The sandwich-like construction confined the organism between the sheets of polymer and avoided release of the fungi or its spores (smallest size ca. 2 μm).[19] Figure 2 shows a cross-section of the test materials

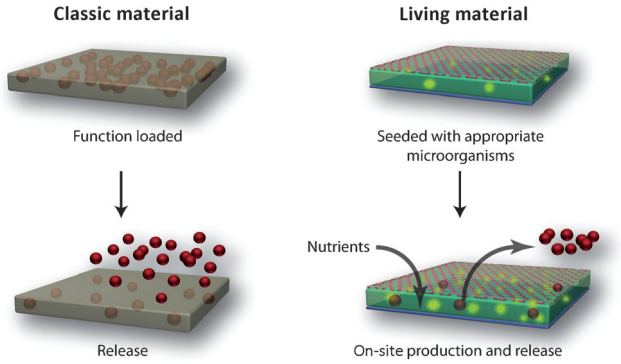


***Figure 1.*** Comparison of a classical with a living material. Whereas a classic active material must be loaded with a specific function (for example silver nanoparticles or penicillin), a living material is seeded with an appropriate microorganism. In a classical functional material, the active ingredients are released (at the latest) during exposure and the activity converges to a minimum. In contrast, the microbes incorporated in the living material produce the active agents on-site and release them. No material supply limits application over a longer time.

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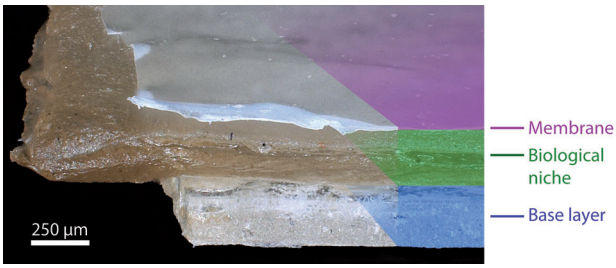


***Figure 2.*** Structure of a living material. Microscopy image of a living material is shown as a cross-section and reveals a layered structure. The living layer provides an artificial biological niche and is enclosed through a base layer (mechanical support) and a porous membrane (400 nm pores). The top membrane allows transport of nutrients and enables the release of penicillin produced in the central living layer to the outside of the sandwich construction.[3]

used herein. The polymer-based physical barriers shield the habitat against invasion of other potentially competing organisms and resulting loss of function. Fungi were shown to remain alive over a prolonged period of time, and proliferated when food was available on its surface, diffusing through the nanoporous top layer.[3] During starvation periods, the fungi go into a hibernation-type state, but they rapidly awake if renewed nutrients become available. Metabolic byproducts (for example $CO_2$) were previously shown to escape efficiently through the nanoporous top layer.[3]

Living surfaces were loaded with fungi at about $10^6$ colony-forming units (CFU) per $cm^2$ and allowed to proliferate for 5 or 10 days before starting penicillin production measurements. The fungi were grown using "top feeding", that is, the growth medium (3 mL) was filled above the nanoporous layer in the beginning of the growth phase (no medium renewal was necessary). Exchanging the top medium with saline (0.9 wt% NaCl in water) and measuring the penicillin concentration by ELISA in the supernatants confirmed production at about 600 ng $mL^{-1}$ within 24 h. After top liquid exchange, penicillin from the living material's biological niche layer underneath (Figure 2) rapidly diffused upwards and refilled the penicillin in the top medium within minutes (Figure 3a, concentrations at 0.05 h and longer). This clearly shows that the nanoporous top layer allows efficient transport of the active ingredient from the site of production (below) to the site of use (above the living material's surface). When *P. chrysogenum* was only allowed to grow for 5 days prior to measurement, these young samples supplied much less penicillin (100 ng $mL^{-1}$). Rapid exchange of the top medium (washout experiments) was performed five times in a row, thus simulating large volume flow above the material. In the case of young living materials (Figure 3b, left, 5 days), only minor amounts of penicillin could be supplied to the top medium. Older living materials (10 days) with more mature fungi showed sustained delivery of at least 200 ng penicillin $mL^{-1}h^{-1}$ for five consecutive runs. On a more quantitative base, in every one hour-long washing cycle, approximately 100 ng penicillin $cm^{-2}$ surface were released (Figure 3b). To put this into a biologically meaningful context: An antibacterial effect on penicillin-sensitive organisms is expected at minimum inhibitory concentrations of around 100 ng $mL^{-1}$.[20]

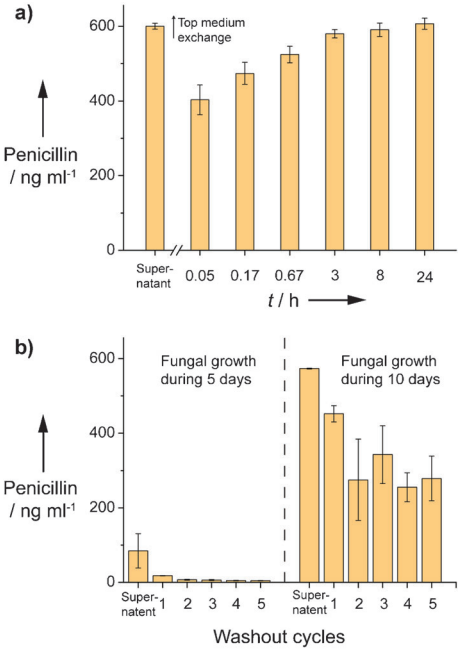


***Figure 3.*** Penicillin production and release by living materials. a) Penicillin production and release of a single 4 $cm^2$ size living material sample. The penicillin of the sandwich compound was rapidly released to liquid (3 mL) residing above the material. b) Washout cycles (1 h/cycle) to test the rate of penicillin production for living materials pre-incubated for 5 and 10 days. A certain maturation and fungi proliferation is needed to sustain sufficient penicillin production.

Direct tests on the penicillin producing surfaces against bacteria were carried out using two controls: We prepared living surfaces with no fungi (that is, negative control with no organism), with *P. roqueforti* (grows very well but produces no appreciable amount of penicillin), and a *P. chrysogenum* strain that produces penicillin. After a 10 day delay phase to allow growth of the fungi, the supernatants were removed, surfaces rinsed, and all three materials were exposed to either bacteria suspensions of penicillin-resistant (Gram-negative) *E. coli* or penicillin-sensitive (Gram-positive) *S. carnosus* (Figure 4). As only *P. chrysogenum* produces penicillin, living materials with those fungi were the only ones to actively inhibit Gram-positive bacteria growth. As Gram-negative bacteria are not responsive to penicillin, *E. coli* was not significantly inhibited when exposed to the produced penicillin from any of the tested living surfaces. The penicillin producing living materials based on *P. chrysogenum,* however, were able to effectively kill planktonic *S. carnosus* within one day. When "empty" or *P. roqueforti*–based living material were brought in contact with bacteria of either Gram type, the bacteria showed standard proliferation.

The fungi within the living material are the key for antibacterial activity and must therefore be maintained alive. A very beneficial aspect of fungi, namely their adaptability, can be exploited. They usually survive environmental changes by remaining in a non-proliferating status or building highly stable spores. In a previous report we have shown how such fungi are indeed able to survive starvation periods.[3] The porous top layer further allows the diffusion of waste products

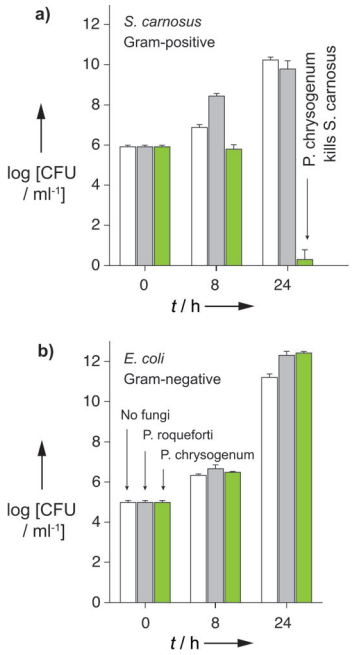


***Figure 4.*** Exposure of Gram-negative and Gram-positive bacteria to various living materials. Two controls ("no fungi" and a "non-penicillin-producing strain") and a penicillin-producing material were investigated and exposed to a) *S. carnosus* and b) *E. coli* contamination. The bacteria proliferated when the living materials only contained *P. roqueforti*, but the Gram-positive bacteria *S. carnosus* died when seeded near a living material containing the penicillin-producing mold *P. chrysogenum*. As additional controls, these surfaces could not inhibit the growth of the penicillin-insensitive control bacterial *E. coli*.

out of the living material and thereby reduces the possibility of self-intoxication. One critical point is the agar medium layer into which the fungi are seeded, as the latter should not dry out completely. Future studies are needed to optimize storage and long-term agar composition and stability.

At the moment, the potential for development of bacterial resistance against the penicillin releasing fungi-containing polymer has not been tested; however, we have to assume that the continuous production of penicillin may ultimately lead to antibiotic resistances. In this regard, it would help to incorporate organisms which produce only antibiotics when sensing the presence of bacteria. Furthermore, the present study only shows antibacterial action in one of the easiest situations (planktonic bacteria). Clinically often more relevant biofilms may however require stronger or different antimicrobial strategies, as such colonies are typically more resistant than single bacteria.

Traditional materials make use of components that are manufactured in large facilities often using a mixture of preparation methods. Functional living materials can however bring stimulus-dependent in situ preparation of active compounds to the application site. We may view traditional functional materials as a way to selectively transport an active compound to a site of application. By contrast, in this work we transport the capability to produce an active compound to the user. Naturally, later strategy is more adaptable. Apart from the previously reported self-cleaning[3] and the antibiotic producing living materials presented herein, plenty of material–organism combinations are conceivable. Depending on the seeded microorganisms, the materials may exhibit several functions and release products that can be harvested above the surface (Supporting Information, Table S1). Making complex response and manufacturing capability as found in microorganisms available to consumer goods design opens unprecedented opportunities to use (bio-) chemical action as part of an object's interaction with users, surrounding organisms, or even inorganic components.

## *Experimental Section*

Microorganisms: *Penicillium roqueforti* (Thom, anamorph deposited as Penicillium roquefortii, ATCC 42294, LGC Standards) was grown in 75 $cm^2$ T-flasks at 21 °C on 50 mL potato dextrose agar (PD, VWR BDH Prolabo) for two weeks before usage. *Penicillium chrysogenum* (Thom, DSM 895, DSMZ Germany) was grown in 75 $cm^2$ T-flasks at 21 °C on 50 mL of culture medium (RM) according to Raper et al.[21] for two weeks before usage. RM was obtained by dissolving 20 g lactose, 9.25 g $MgSO_4 \cdot 7H_2O$, 0.5 g $KH_2PO_4$, 3.0 g $NaNO_3$, 40 mL corn step liquor (Sigma-Fine Chemicals), and 1.2 g glucose in 925 milliliter water ($ddH_2O$, Millipore, 18.2 MΩ cm at 25 °C) and subsequently autoclaved 15 min at 121 °C. *Escherichia coli* (strain C43) and *Staphylococcus carnosus* (strain TM300) were grown in Difco™ LB broth (Chemie Brunschwig) for 4 h at 37 °C and gently agitated to a concentration of about $10^8$ CFU $mL^{-1}$ (colony forming units per milliliter). This suspension was diluted to the required concentration with physiological saline (0.9 wt % NaCl in water).

Preparation of living materials: By the aid of acetone, cell culture inserts (Millipore, PIHP03050, diameter 31.5 mm, polycarbonate membrane, pore size 400 nm) were adhered to standard 6-well plate covers (1.5 mm borehole for food supply). 1 mL of liquefied agar (15 g agar (AppliChem) in 985 mL water ($ddH_2O$, Millipore, 18.2 MΩ cm at 25 °C), autoclaved 15 min at 121 °C) containing no additional fungi, 0.1 mL *P. roqueforti* suspension, resp. 0.1 mL *P. chrysogenum* suspension was poured on the bottom of the cell culture inserts (for 6 well plates, 4.5 $cm^2$/well). The fungi suspensions were obtained by high-shear mixing (IKA, Ultra-Turrax T10, 30000 rpm) an eighth of a fully grown fungi culture in 50 mL $ddH_2O$ for 1 min. After gelification for 2 h at ambient conditions, a thin layer of an acrylate polymer (Dupli-Color, Aerosolart) was sprayed onto the living layer. After 5 h drying the samples were turned over, covered with 3 mL RM, and incubated at ambient conditions for 5 resp. 10 days.

Colony-forming unit (CFU) determination: To quantify the amount of bacteria on the living materials surfaces dilution rows in saline (0.9 % NaCl in water) up to $10^{-10}$ of the supernatants were plated in duplicate on CASO tryptic soy agar contact plates (VWR) and incubated at 37 °C before readout. To quantify the CFU of fungi within living materials the membrane and base layer were removed before high-shear mixing (IKA, Ultra-Turrax T10 30000 rpm) the samples in 20 mL $ddH_2O$ for 1 min. Dilution rows up to $10^{-8}$ were plated on PD and incubated at room temperature before readout. A detailed procedure and validation of the method was published earlier.[3]

For the quantitative measurement of the penicillin content in the solutions on the living materials surfaces an enzyme immunoassay (PEN-E01, Immunolab GmbH, Germany) was utilized. Briefly, an immobilized penicillin conjugate on the microtiter plate and penicillin within the sample competed for the binding sites of added antibodies. After the treatment with a peroxidase, a substrate solution and the addition of a stop solution, the absorbance was measured at 450 nm on a Tecan infinite F200 plate reader. To determine the amount of

  

penicillin that can be washed out (Figure 3), the supernatants were removed and the surfaces covered with fresh saline each time.

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