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Temperature-Responsive Polymer Brushes Switching from Bactericidal to Cell-Repellent

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Materials exhibiting antibacterial properties at room temperature and turning biocompatible and non-adhesive for in vivo conditions, are extremely attractive for devices that have to be ultimately introduced in living beings. Indeed, infections related to the use of invasive biomedical and medical items are still one of the main medical complications that cause high rates of mortality.[1] Despite sanitation protocols, a well-identified route for patient bacterial infection is transmission through contaminated instruments such as intubation tubes, catheters, surgical drains or endoscopes that bypass the natural protective barriers of the body.[1]

One way to prevent this transmission would be by grafting on the surface of the material a thin responsive coating able to switch from bactericidal for ambient storage conditions, to passive in vivo. Smart surfaces exhibiting stimuli-responsive properties are well-known, and have been used recently to fabricate temperature-switchable surfaces able to modulate biomolecular activity.[2] Such systems are invariably based on thermoresponsive polymer coatings, which are swollen by water and exhibit anti-fouling properties below a collapse transition temperature T_{coll}, while turning bio-adhesive above T_{coll} due to the expulsion of water. A seminal example of such coatings are brushes of poly(*N*-isopropylacrylamide) (PNIPAM),[3] a polymer which exhibits a lower critical solution temperature (LCST) in a physiologically-relevant range of temperature,[4] but was recently described as a slightly cytotoxic.[4c] However, for all systems reported to date, the surface switches from cell-repellent to cell-adherent upon increasing temperature. This is in marked contrast with the present work, which aims at developing a coating able to repel cells at physiological temperatures, while killing them at lower ones.

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A more recent example of thermoresponsive coating are brushes of copolymers based on 2-(2-methoxyethoxy)ethyl methacrylate (MEO_2MA) and oligo(ethylene glycol) methacrylate (OEGMA) (**Figure 1**).[5,6] These random copolymers exhibit a LCST less sensitive to surrounding conditions than PNIPAM and are essentially based on biorepellent and bioinert oligo(ethylene) segments.[7] Recently, we have shown that the average collapse temperature of these brushes can be finely adjusted from 22 °C to 40 °C by tuning their content in OEGMA and the length of the OEGMA side chains.[5a,b] Importantly, we also showed that the collapse of such brushes occurs over a temperature range of about 15 °C, progressing from the bottom to the top surface of the brush.[5b] This offers tremendous opportunities to design advanced surfaces capable to exhibit a more complex behavior than simply switching from non-adhesive to adhesive with temperature, provided the brushes are properly designed. Here, we take advantage of this complexity to turn a material surface from bactericidal at room temperature to non-adhesive and bioinert at 38 °C.

The most efficient way to prepare biocidal coatings is to incorporate in them antibacterial compounds. Various substances based on metal or quaternary ammonium salts or antibiotics were extensively tested for the preparation of

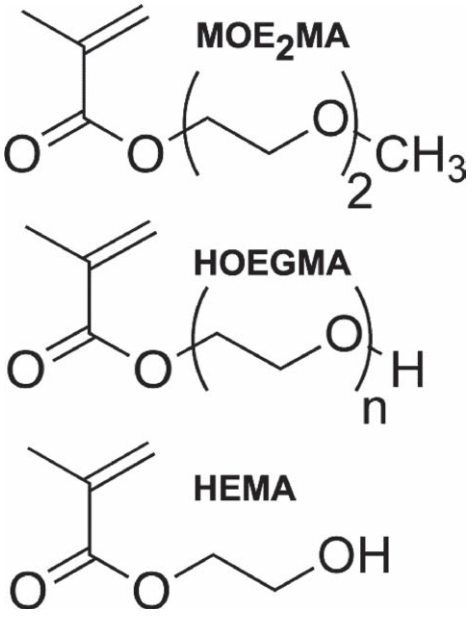

Figure 1. Monomers used for the preparation of thermoresponsive copolymer brushes.

antibacterial layers.[8] However, they are not completely satisfying regarding their potential impact on the environment, their limited efficiency or their implication in the emergence of resisting bacteria.[9] Recently, antimicrobial peptides (AMP's) have emerged as a promising new class of bactericidal substances.[10] Compared to conventional synthetic antimicrobial drugs, these natural compounds that kill bacteria by penetrating cell membranes and making them permeable,[10c] have the advantage to act at a lower concentration and to have a broader spectrum of antibacterial and even antifungal activity, while limiting pathogen resistance.[10b] An interesting example of bactericidal coating based on an antibacterial peptide is provided by some living organisms such as amphibians that are continuously exposed to bacterial attacks. These organisms have developed highly efficient strategies to counter bacterial adhesion, by secreting a thin antibacterial skin mucus containing AMP's to protect themselves.[10a] The development of synthetic layers directly bio-inspired by this strategy is highly attractive, particularly for biomedical applications. However, the in vivo use of AMP-based coatings to protect medical items might be limited by the frequent hemolytic activity of natural AMP's. Therefore, designing a coating that can kill bacteria at room temperature, while hiding AMP's and preserving antifouling properties at physiological conditions, is not only scientifically challenging but also practically useful.

Here, thermoresponsive copolymer brushes based on MEO_2MA, hydroxyl-terminated oligo(ethylene glycol) methacrylate (HOEGMA of 360 g mol^{-1} molar mass) and 2-hydroxyethyl methacrylate (HEMA) (Figure 1) were grown by Atom Transfer Radical Polymerization (ATRP) from silicon surfaces. An antimicrobial peptide, magainin-I active against Gram-positive and Gram-negative bacteria,[11] was grafted on the hydroxyl groups of the brush as described before.[12] Various compositions of the monomer mixtures were tested in order to prepare thermoresponsive functional brushes exhibiting a collapse transition temperature of the bulk of the brush, T_{coll}, very close to the physiological temperature. T_{coll} was determined by quartz crystal microbalance with dissipation monitoring (QCM-D) measurements in water, and corresponds to the collapse of about 50 % of the bulk of the brush as demonstrated before.[5b] Compared to a brush of pure poly(MEO_2MA) which has a T_{coll} of 22.2 °C,[5b] increasing the content of the brush in the more hydrophilic HOEGMA raises T_{coll}, whereas the addition of HEMA moieties tends to decrease T_{coll} (**Figure 2**). Hence, T_{coll} can be finely tuned by adjusting the composition of the monomer mixture, while independently setting the amount of reactive –OH groups included in the brush. Importantly, equilibrium contact angle measurements have shown that the outermost chain segments of oligo(ethylene glycol) methacrylate-based brushes only collapse at a temperature T^S_{coll}, higher by about 8 °C than the collapse temperature of the bulk of the brush, T_{coll}.[5b] This difference, which is also predicted on theoretical grounds,[13] will be advantageously used here to have a partially collapsed brush whose outer surface layer is still able to resist bacterial adhesion over the temperature range of interest.

A poly(MEO_2MA_{50}-*co*-$HOEGMA_{20}$-*co*-$HEMA_{30}$) with T_{coll} = 35 ± 1 °C and containing 50 % of hydroxyl-reactive side-chains, was selected to prepare switchable bactericidal layers. The antibacterial peptide MAG-Cys derived from magainin-I and bearing an additional *C*-terminal cysteine residue was grafted onto hydroxyl functions of the copolymer brushes through a *N*-(*p*-maleimidophenyl)isocyanate (PMPI) heterolinker according to a procedure described previously.[12] Quantitative XPS measurements performed before on similar brushes have shown that from 5 to 10% of available hydroxyl moieties are substituted by the magainin peptide.[12] This most probably occurs preferentially at the external surface of the brush due to the size and the nature of the peptide.

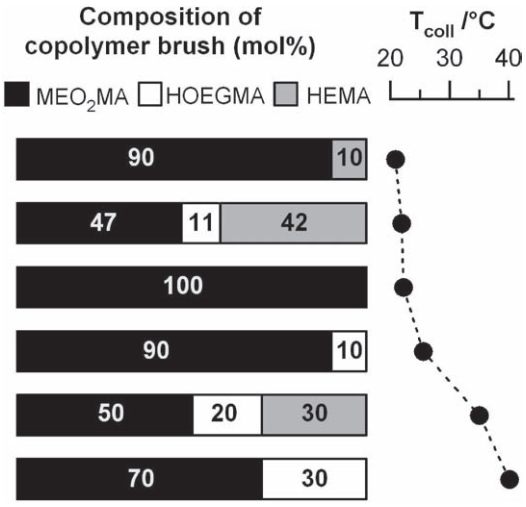

Figure 2. Collapse transition temperature (T_{coll}) of P(MEO_2MA_x-$HOEGMA_y$-$HEMA_{(1-x-y)}$) brushes in water.

The accessibility of the immobilized peptide was checked as a function of temperature, by first grafting a biotin-tagged magainin derivative (Biotinyl-MAG-Cys). The resulting sample was subsequently incubated with fluorescent streptavidin^{FITC} at 26 °C, well below T_{coll} (35 °C), or slightly above T_{coll} at 38 and 42 °C. The presence of streptavidin^{FITC} immobilized on Biotinyl-MAG-Cys-funtionalized brushes is detected at all temperatures by fluorescence microscopy (**Figure 3**). However, a large decrease of the fluorescence of the brush surface is observed when the sample is incubated above T_{coll}. The decrease of the fluorescence intensity is ~40 % between 26 and 38 °C and ~60 % between 26 and 42 °C, showing that the accessibility of the grafted peptide towards the relatively large streptavidin molecules decreases progressively above T_{coll}. This is because, at lower temperatures, the extended and swollen conformation of the copolymer chains promotes the accessibility of the biotin-tagged peptide. By contrast, at a temperature higher than T_{coll}, the progressive collapse of the brush provides a more hydrophobic environment into which amphipathic peptide molecules tend to bury (Figure 3), which considerably limits the accessibility of the biotin-tagged moieties.

However, the binding between the biotin-tagged peptide and streptavidin^{FITC} is not completely suppressed at 42 °C, since residual fluorescence is still detected at 42 °C (Figure 3). This is because the outermost segments of the responsive copolymer chains are not completely collapsed at this temperature, as mentioned above and in agreement with our previous report showing that the surface of the brush collapses at a higher

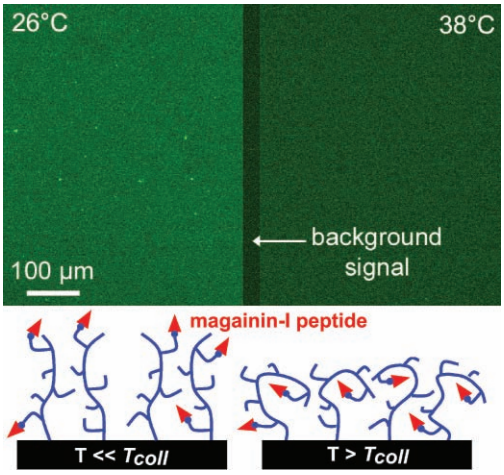

Figure 3. Fluorescence images of (Biotinyl-MAG-Cys)-grafted $P(MEO_2MA_{50}$-$HOEGMA_{20}$-$HEMA_{30})$ brushes incubated at 26 and 38 °C in streptavidin^{FITC} solution (top), and schematic drawing of the brush conformation well below and slightly above T_{coll} (bottom). To avoid different imaging histories, both samples were placed on a microscope glass slide and imaged simultaneously under the microscope, with the glass substrate providing the background fluorescence level.

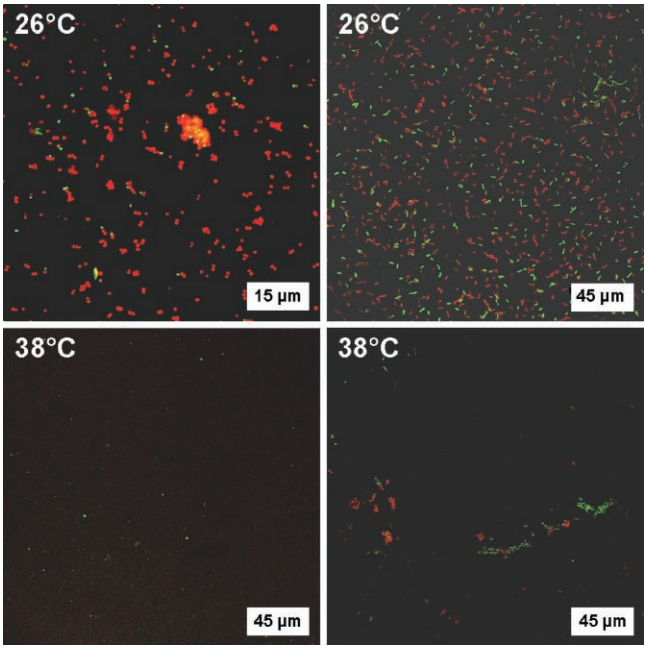

Figure 4. (MAG-Cys)-functionalized $P(MEO_2MA_{50}$-$HOEGMA_{20}$-$HEMA_{30})$ brush incubated in the presence of *L. ivanovii* (left) or *E. coli* (right) and subsequently stained with the LIVE/DEAD viability kit; samples incubated at 26 °C (top) and 38 °C (down).

temperature than the bulk of the brush.[5b] Hence, complete hiding of the peptide to streptavidin is not achieved at 42 °C.

The responsive brushes, functionalized or not by the magainin peptide, were then tested against two strains of Gram-positive and Gram-negative bacteria, *L. ivanovii* and *E. coli*, respectively. The bacterial assays were performed at 26 and 38 °C to investigate the influence of the thermal transition of the copolymer brushes on the bactericidal properties of the samples. Experimentally, the brushes were incubated for 3h at a given temperature in bacteria suspensions (10^7 Colony Forming Units–CFU mL^{-1}), then gently rinsed at the same temperature. Subsequent staining was performed with the fluorescent LIVE/DEAD viability kit to check the viability of the bacteria adhering on the sample surface.

When the assays were performed on the magainin-free brushes, no bacteria could be detected on the surfaces by confocal laser scanning microscopy (CLSM) for both tested temperatures, showing that the cell-repelling property of the brush surface is preserved up to 38 °C for both strains. It should be noted that a previous report on similar brushes also demonstrated complete bacterial repellence below T_{coll}.[12] Here, the cell-repellence is also kept at 38 °C, a temperature slightly higher than T_{coll} (= 35 °C), due to the higher collapse temperature T^S_{coll} of the surface compared to bulk of the brush,[5b] as mentioned above. In addition, experiments of bacterial culturability performed at 25 °C in the presence of silica microbeads coated with a similar brush confirmed that oligo(ethylene oxide) methacrylate-based brushes do not affect the viability of the cells (Table 2, Supporting Information). Thus, the brushes used here can safely be considered as cell-repellent and bio-inert up to 38 °C.

In contrast, magainin-functionalized brushes display a completely different behavior. Indeed CLSM overlay images recorded at both temperatures for *L. ivanovii* and *E. coli* assays (**Figure 4**) show a large fraction of dead (i.e. red-colored) adhering cells on both samples incubated at 26 °C, below T_{coll}. Compared to unfunctionalized brush surfaces which are cell-repellent, the presence of bacteria on the peptide-functionalized brushes results from the supplementary interaction established between the peptide and bacterial membranes. Moreover, the fact that all bacteria seen on the surface are dead indicates that the grafted peptide is accessible enough in the swollen brush at 26 °C to induce bacterial lysis. Hence, our observations support the notion that, below T_{coll}, the peptide interacts with the bacterial membrane, anchors the bacteria on the surface and kills them. It has to be noticed that an extensive additional rinsing of the samples as well as an additional incubation in water at 38 °C do not allow to remove the adhered dead bacteria from the brush surface. These observations prove the existence of a strong irreversible interaction between the magainin molecules and the bacteria membrane at 26 °C. It is however difficult to quantify precisely the degree of antibacterial activity of the magainin-functionalized brush by criterion such as the "log kill"[8f] since the reference ungrafted brush prevents bacterial adhesion as mentioned above.

When the incubation temperature is increased to 38 °C above T_{coll} but still below T^S_{coll}, no *L. ivanovii* cells are seen on the brush surface whereas only a few red and green *E. coli* aggregates are detected sparsely (Figure 4). This shows that the surface properties of the peptide-functionalized brushes have changed from dominantly bactericidal at 26 °C to dominantly non-adhesive when the temperature becomes slightly higher than T_{coll}. At this temperature of intermediate collapse, the peptide-functionalized brushes thus behave as simple cell-repellent non-functionalized brushes. Considering the

previously reported high bactericidal activity of magainin immobilized on surfaces at both 25 °C and 37 °C,[12,14] the suppression of the bactericidal activity of our peptide-functionalized brushes cannot be attributed to the influence of temperature on peptide activity. Moreover, it was previously reported that pristine copolymer brushes based on poly(oligo(ethylene glycol)methacrylate) have a strong ability to attach or to repel living cells depending on external temperature.[6] Indeed, at temperatures significantly above T_{coll}, the collapsed brush surface favors cell adhesion. By contrast, at temperatures lower than T_{coll}, the solvated oligo(ethylene glycol) segments tend to repel the surrounding cells. However, as stated before, brushes of thermoresponsive polymers do not exhibit a sharp transition from an extended to a collapsed state with temperature, but have their inner segments collapsing before their outer segments.[5b] As a consequence, slightly above the collapse transition of the bulk of the brush, the external segments of the anchored macromolecules are still sufficiently swollen to provide cell-repellent properties to the surface. Part of the magainin molecules are not more accessible as demonstrated by the experiments performed with the streptavidin^{FITC}. These peptide molecules are most probably buried in more compact hydrophobic regions within the brush, and therefore do not act against bacteria. The remaining peptide molecules, which are still accessible to streptavidin^{FITC}, are also not active against bacteria. This can be explained by the combination of two factors. First, the collapsing chains reduce the longer range mobility of the peptide molecules, preventing them to interact properly with bacterial membranes. Nevertheless, the shorter range mobility of the peptide molecules is still preserved in the outer part of the brush which is not yet collapsed, thereby allowing the peptides to rearrange spatially in order to decrease the free energy of the system. This can be achieved by accumulating short hydrophilic chain segments at the outermost surface of the brush, whereas the amphipathic peptide molecules tend to accumulate slightly deeper in the more hydrophobic environment offered by the collapsing brush (Figure 3). This configuration will prevent bacterial lysis but not streptavidin binding, as observed here.

The development of such a surface which switches from cell-killing to cell-repellent by increasing temperature fully differs from previous works usually showing surfaces switching from cell-repellent to cell-adherent when temperature increases.[2,6,15] Obviously, heating further the peptide-functionalized brush would lead to the ultimate collapse of its outer segments, and therefore to the adhesion of bacteria on its surface; however, this range of temperature is detrimental to the bacteria and outside physiological conditions, and was therefore not explored here.

In conclusion, we have developed novel smart coatings which can switch reversibly between bactericidal and bacteria-repellent surface properties, mediated by an external thermal stimulus. A proper adjustment of the composition of the thermoresponsive oligo(ethylene glycol) methacrylate-based copolymer brush allows us to control the presentation of an antibacterial peptide at a temperature very close to the physiological temperature. The proof-of-concept demonstration of our study could be advantageously extended to show or hide various drug molecules and to prepare new layers with switchable bioactivity, which should be of interest for applications in the field of medicine.

Experimental Section

Materials: Di(ethylene glycol) methyl ether methacrylate (MEO_2MA) (95%), hydroxyl-terminated oligo(ethylene glycol) ether methacrylate (HOEGMA) of molar mass ~ 360 g mol^{-1} and 2-hydroxyethylmethacrylate (HEMA) were obtained from Aldrich and were used without purification. FITC-labelled streptavidin (streptavidin^{FITC}) and *N*-(p-maleimidophenyl) isocyanate (PMPI) were purchased from AbD Serotec and Appolo Scientific respectively. Copper(I) chloride (99.995+%) (CuICl), copper(II) chloride (99.999+%) (CuIICl2) and 2,2'-dipyridyl (99+%) (bipy), tris(hydroxymethyl)aminomethane (tris) were from Aldrich. The 2-bromo-2-methyl propionic acid 3-trichlorosilanyl-propyl ester silane initiator was synthesized according to a previously published procedure.[16] All solvents were distilled before use, except methanol which was analytical reagent grade. Milli-Q water (resistivity higher than 18.2 MΩ cm) was obtained from a Millipore Simplicity 185 system. One-side polished (100) silicon wafers (<100> orientation) were obtained from ACM (France); they were cleaned by treatment in a hot piranha solution (H_2O_2 (35%) : H_2SO_4 (98%) 1:1 v/v) for 20 min (caution: piranha solution is extremely corrosive) and then thoroughly washed with pure Milli-Q water before use. Quartz crystal sensors covered with a layer of SiO_2 (QSX 303) were purchased from Q-Sense (Sweden); they were rinsed with ethanol and cleaned by UV/ozone treatment for 30 min before use. Magainin-I derivatives with an additional C-terminal cysteine residue and tagged (Biotinyl-MAG-Cys) or not (MAG-Cys) with a *N*-terminal biotin moiety were synthesized by the solid phase methodology as described previously.[10] LIVE/DEAD BacLight Bacterial Viability Kit L7007 containing SYTO 9 and propidium iodide dyes was purchased from Molecular Probes.

Preparation of copolymer brushes: Brushes were grown according to a previously published protocol on silicon wafers or on QCM sensors.[5a] Briefly, the substrates were silanized with an ATRP silane initiator 2-bromo-2-methyl propionic acid 3-trichlorosilanyl-propyl ester as described previously.[17] Then, different series of copolymer brushes were synthesized by controlled radical polymerization using mixtures of MEO_2MA, HOEGMA and HEMA of various compositions in the feed solution while keeping the total molar content in methacrylate moieties constant. For this, initiator-grafted substrates were immersed in the oxygen-free feed solution for a given time. Then the samples were removed, washed with water then methanol, dried with a stream of nitrogen and stored under nitrogen before use. The polymerization times used for each series were adapted to get copolymer brushes with an average thickness of about 100 nm measured by ellipsometry. The samples poly(MEO_2MA_x-*co*-$HOEGMA_y$-*co*-$HEMA_{(1-x-y)}$) synthesized from various monomer compositions are summarized in Supporting Information (Table S1).

Magainin grafting on copolymer brushes: The magainin-I derivatives MAG-Cys and Biotinyl-MAG-Cys with an additional C-terminal cysteine residue were grafted on hydroxyl groups of poly(MEO_2MA-*co*-HOEGMA-*co*-HEMA) (50:30:20) brushes via a PMPI heterolinker as described previously.[12]

Optical Fluorescence Microscopy: Fluorescence images were taken at room temperature with a Leica MDR microscope equipped with a FITC filter set and an Epi-Fluorescence module, and connected to a C4742–80-12AG Hamamatsu CDD camera.

Confocal Laser Scanning Fluorescence Microscopy (CLSM): CLSM imaging was carried out at room temperature with a Leica SP2 upright confocal laser scanning microscope (DM RXA2) equipped with an Acousto-Optical Beam Splitter (AOBS) system and using a 63 × oil immersion objective with numerical aperture 1.32 (Leica Microsystems, Rueil-Malmaison, France). SYTO 9 was excited at 476 nm and observed from 485 to 540 nm and propidium iodide was excited at 514 nm

and observed from 580 to 670 nm. The laser power, the gain and the offset for each photomultiplier have been adjusted to optimize bacteria detection.

Images of the CLSM observations (1024 × 1024 pixels) have been acquired through sequential mode to avoid fluorescence emission spectrum overlap and the signal-to-noise ratio has been increased through line (×2) and frame (×4) averaging. Overlay images have been built with post acquisition Leica Confocal Software (LCS).

Collapse transition temperature from Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D): QCM-D measurements were performed in water with a Q-Sense E4 microbalance.[5] Data collection was realized in two ways, static and dynamic. For static measurements, the system temperature was increased by steps and equilibrated for at least 50 min at each step before performing the acquisition. For dynamic measurements, the temperature was ramped at a rate of 0.2 °C/min while continuously acquiring data. The collapse transition temperature was determined by analyzing the variation of the shift of resonance frequency with temperature, $\Delta f(T)$. The frequency shift increases continuously with temperature but exhibits different slopes in the probed temperature range, due to the collapse of the brush with its associated decrease of brush height, loss of water, and change in viscoelastic properties. The inflection point of $\Delta f(T)$ allows to determine the temperature of the collapse of half the brush (computed numerically after polynomial smoothing to remove noise), as fully discussed in the Supporting Information and elsewhere.[5b]

Bacterial inhibition assays: Magainin-functionalized copolymer brushes were tested against Gram positive *Listeria ivanovii* (strain kindly provided by the UMR CNRS 6008 of University of Poitiers, France) and Gram negative *Escherichia coli* (strain K12, kindly provided by the Laboratoire de Microbiologie du Froid, Evreux, France). Both bacteria strains were precultured in Luria-Bertani (LB) broth (DifcoTM) for 15 h at 36 °C then harvested by centrifugation (3000 × g for 15 min) and resuspended in distilled water. A functionalized substrate was immersed in 10 mL of diluted bacteria suspension freshly prepared in distilled water (10^7 Colony Forming Units- CFU mL^{-1}). After 3 h incubation at a given temperature the sample was taken out and briefly rinsed with distilled water at the same temperature. To test the viability of bacteria adhering on the functionalized copolymer brush, the cells were immediately stained with the LIVE/DEAD BacLight Bacterial Viability Kit. For this, the wet substrate was immersed in 2 mL of diluted viability kit solution. After an incubation time of 30 min, the sample was briefly rinsed with distilled water and the surface was visualized by CLSM. The viability of the cells suspended in the distilled water was also controlled by counting the number of bacteria colonies grown on a seeded Plate Count Agar medium (DifcoTM). This control experiment confirmed that the viability of the bacteria was absolutely non affected after an incubation time of 3 h in water at 26 and 38 °C.

Supporting Information

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

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