

Research Article

Ink-Jet Printing: Perfect Tool for Cantilever Array Sensor Preparation for Microbial Growth Detection

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The use of microcantilever arrays for microbial growth detection provides a rapid and reliable technique for monitoring growth in industrial and clinical applications. Improving the reproducibility and sensitivity of this technique is of great importance. Ink-jet printing has been successfully used in microfabrication and biofabrication due to its high precision; however, only a few microbe-based applications have been reported. Here we demonstrate the advantages of its use for microcantilever based-growth sensing. Four microbial strains *Escherichia coli*, *Staphylococcus aureus* subsp. *aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* were deposited and successfully grown on agarose-coated cantilevers by ink-jet printing. When compared to the capillary-coating method, ink-jet printing demonstrated more controlled cell deposition on cantilevers. The effect of various conditions on cell morphology was also investigated.

1. Introduction

The bioengineering fields of genomics, drug screening, tissue engineering, regenerative medicine, and various biosensor applications rely on biofabrication techniques which combine living and nonliving components deposited in a controlled manner. Bioprinting provides a rapid and reliable alternative to traditional methods giving the required spatial resolution for these applications [1]. Although biological structures are considered fragile, many studies have proven that ink-jet printing is feasible for a broad spectrum of biological material [2–6], and even more complex living systems such as cells can be deposited by this technique [7, 8]. Drop-on-demand bioprinters use different mechanisms to force the “bio-ink” through a microfluidic chamber to the output orifice [1]. Thermal ink-jet printheads use a heating element to raise the temperature in the reservoir creating a bubble which then forces a small amount of ink through the output orifice. In a piezoelectric printhead, a piezo actuator is supplied with short electrical pulses which are converted into short pressure pulses. These pulses propagate through the liquid. Due to inertia forces and supported by surface tension, a small volume of liquid breaks off the liquid

column to form a droplet. The droplet then flies freely with a velocity of 2–3 m/s. A pressure-driven printhead uses a pressure source behind the reservoir to produce a force on the liquid. A gate opens to allow ink to flow through the orifice [1]. It is clear that each of these approaches could damage the biomaterial by heat, electric field gradient, or pressure shock, and therefore, the range of working parameters must be optimised. Depending on the application, other issues such as nozzle clogging by cells or particles, aseptic printing, and aerosol formation have to be taken into account. Despite these problems, the spatial precision of the printed biomaterial renders these techniques highly advantageous for biosensing applications. Computer-controlled deposition offers a rapid functionalisation technique for micron-sized sensors such as cantilevers, quartz crystal microbalance (QCM), or surface plasmon resonance- (SPR) based sensing.

In this paper, we focus on microcantilever array functionalisation for cell-based sensor applications. Cantilever arrays operated in dynamic mode allow microbial growth analysis of various microbial cultures faster than conventional culturing techniques [9, 10]. This biological sensor is based on oscillating cantilevers, where additional mass loading onto the cantilever surface results in a change of its

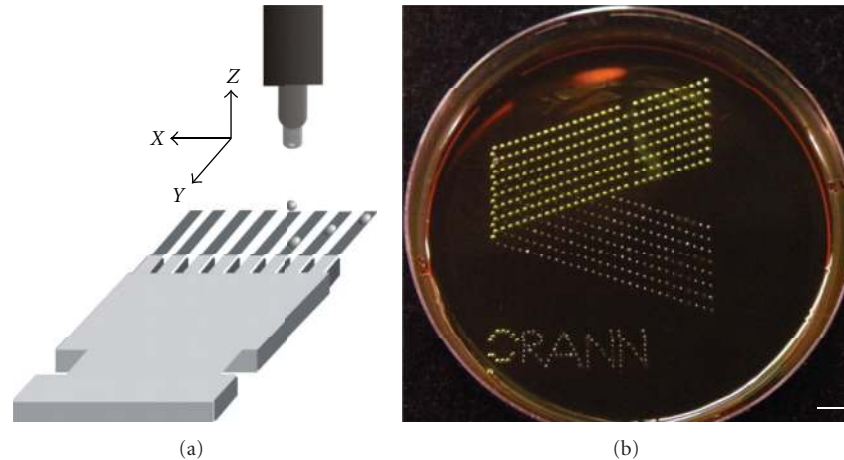


FIGURE 1: (a) Illustration of autodrop ink-jet spotting system for cantilever array functionalisation. Piezo-driven micropipette is mounted on a positioning system which has a positional accuracy of $1\ \mu\text{m}$ in all directions. (b) Viable *E. coli* cells are able to be inoculated by spotting on an agar plate in a controlled manner. The developing colonies form the CRANN Logo after an incubation of 18 hours at 35°C . Scale bar 5 mm. The different optical appearance of the individual *E. coli* colonies spotted is a result of different rates of metabolism which lead to different colouring.

resonance frequency. Ramos et al. demonstrated [11] that the response of nanomechanical resonators depends on both the position and the stiffness of adsorbed biological material on the cantilever surface. This is important for the achievable sensitivity of the technique. Here we introduce the use of ink-jet printing for the functionalisation of cantilevers with industry-relevant microbial species for growth detection.

2. Materials and Methods

2.1. Chemicals, Cultures. Chemicals and culturing media were purchased from Sigma Aldrich (Arklow, Ireland) unless otherwise stated. The following microbial strains were obtained from Collection de l'Institut Pasteur (Paris, France): *Escherichia coli* CIP 53.126 (*E. coli*), *Pseudomonas aeruginosa* CIP 82.118 (*P. aeruginosa*), *Staphylococcus aureus* subsp. *aureus* CIP 4.83 (*S. aureus*), and *Candida albicans* CIP 48.72 (*C. albicans*). Bacterial and *C. albicans* strains were maintained on LB (0.5% NaCl, 0.5% yeast extract, 1% tryptone) or potato dextrose agar (PDA) slants, respectively, and stored at 4°C . Overnight cultures were prepared (200 rpm, 35°C , 15–18 h) from single colonies of each organism. The following day, 1 mL of the overnight cultures was transferred into 30 mL of LB or malt extract broth (ME) for bacterial or fungal cultures respectively, and cultured (200 rpm, 35°C) for 110 min in order to reach a logarithmic growth rate. One mL of each culture was precipitated by centrifugation (10 000 rpm, 5 min, Genofuge 16 M, Techne) and resuspended in 0.5 mL of the tested medium (LB, 10% LB, ME, 10% ME, or deionised water). Cell concentrations of the cultures were determined using cell counting chambers and optical microscopy.

2.2. Cantilever Preparation. Cantilever arrays were obtained from the IBM Zurich Research Laboratory (Zurich, Switzerland). Each array has 8 cantilevers with a length of $500\ \mu\text{m}$,

width of $100\ \mu\text{m}$, and thickness of $2\text{--}7\ \mu\text{m}$ with a pitch of $250\ \mu\text{m}$. In preparation for microbial coating, the arrays were cleaned using O_2 plasma (0.3 mbar, 3 min, PICO Barrel Asher: Diener electronic GmbH and Co. KG, Ebhausen, Germany) and silanised for 45 min (1% (3-glycidyloxypropyl)-trimethoxysilane, 1% N-ethyl-diisopropylamine in water-free toluene). The epoxy-silanised arrays were washed in water-free toluene (2×15 min) and dried in N_2 and subsequently coated with 1% (wt/vol) agarose-water solution (SeaKem Gold Agarose, Bioconcept; NH, USA) by using preheated ($>100^\circ\text{C}$) glass microcapillaries (King Precision Glass Inc., Calif, USA). The pH of the melted agarose was adjusted to pH 11.9 by adding 2 M NaOH to the solution. The cantilevers were incubated in the agarose-filled capillaries for 5 seconds. Using this method a thin agarose layer is formed on the cantilever surface. The boundary of this gel layer is visible on the images of each cantilever.

2.3. Capillary Coating. As a control experiment, cantilevers were functionalised with microbes using glass microcapillaries. Agarose-coated cantilevers were immersed to full, half and 1/5 of their lengths for 6, 4, and 2 minutes, respectively, (Figure 2(a)). Capillary tubes were loaded with *E. coli* suspension (10% LB, 2.49×10^8 cells/mL).

2.4. Ink-Jet Spotting. The MD-P-801 autodrop dispensing system (Microdrop, Norderstedt, Germany) is comprised of a piezo-driven pipette (AD-K-501) and is mounted on a three axis micropositioning system which has an accuracy of $1\ \mu\text{m}$ in all directions (Figure 1). The nozzle diameter used was $50\ \mu\text{m}$. The volume of liquid deposited is not only dependant on its viscosity but also on the voltage and the pulse length applied on the piezo actuator. In order to deposit one droplet per pulse, these parameter settings have to be adjusted for each individual suspension prior to

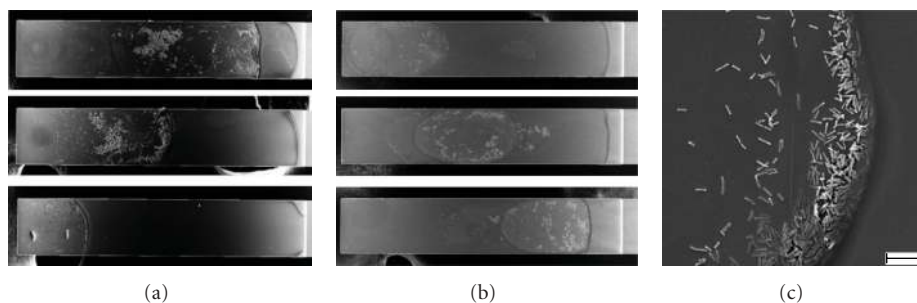


FIGURE 2: (a) SEM images of capillary-coated cantilevers for full (top), half (medium), and 1/5 (bottom) immersion in *E. coli* suspension. (b) Ten drops of *E. coli* suspension were deposited at different positions along the cantilevers' longitudinal axis. Cantilever arrays in images (a) and (b) were incubated for 24 h at optimal growth conditions before SEM imaging. The width of the cantilevers is $100\ \mu\text{m}$. The used suspension (2.49×10^8 cells/mL) was prepared in 10% LB. (c) SEM image showing accumulation of *E. coli* cells at the front end of capillary tube after functionalisation. Scale bar $10\ \mu\text{m}$.

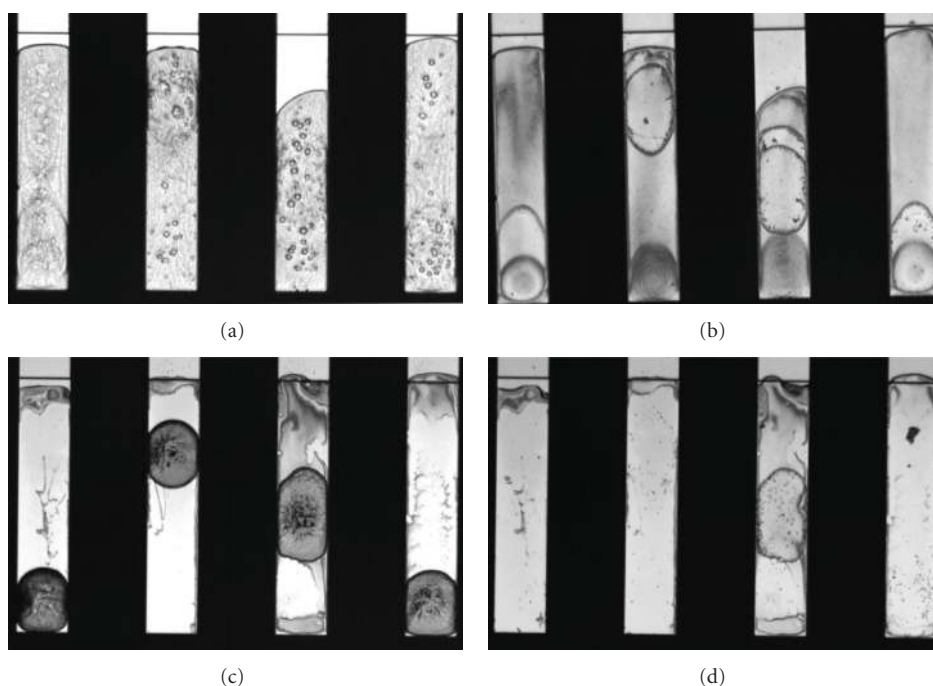


FIGURE 3: Optical microscope images of cantilevers with widths of $100\ \mu\text{m}$. Ten drops of *E. coli* suspension (2.49×10^8 cells/mL) were deposited at different positions on the cantilevers. (a) Cantilever array was incubated at growth conditions before image was taken. (b) Array was incubated at growth conditions then glutaraldehyde fixation (see Materials and Methods section) of the sample was carried out. (c) Array was dried at room conditions after deposition of cells (nongrowth control) (d) Array was prepared as a nongrowth control, then glutaraldehyde fixation was carried out.

deposition. The final spot diameter depends on the surface tension of the printed material and on the surface on which it is deposited. By using a low pulse frequency in our experiments we allowed each droplet to soak into the agarose surface before the next droplet landed on the surface. With this method, aerosol formation could be prevented, and; therefore, no cross-contamination between the cantilevers is expected. The micropipette was cleaned 3 times with 70% ethanol solution and twice with 96% ethanol solution before use. These cleaning steps were sufficient to avoid nozzle clogging or cross-contamination. Cell suspensions prepared as mentioned previously were loaded into 96-well

microtiter plates (Sterilin Ltd., Norwick, UK). Prior to filling the micropipette, solutions in the microtiter plate reservoirs were mixed in order to provide an even cell concentration in the nozzle. Droplets of the different suspensions were spotted on each cantilever independently. To avoid cross-contamination of the lever surfaces the nozzle was cleaned with ethanol between each tested suspension.

2.5. Scanning Electron Microscopy (SEM) Sample Preparation. The cell-functionalised cantilever arrays were exposed to relatively dry room conditions (20–40% RH) for less than 30 minutes. For growth testing, they were placed in high

TABLE 1: Calculation of drop volume after deposition of 180 droplets of microbe suspensions.

Cells/mL	Total cell number/180 drops	Drop volume (L)	Average cell number/drop
6.3×10^6	226	1.99×10^{-10}	1.25
1.175×10^6	45	2.13×10^{-10}	0.25
$\sim 6.3 \times 10^5$	18	1.59×10^{-10}	0.1

humidity (>95% RH) at 35°C. For nongrowth controls, they were left to dry completely at room conditions. High humidity during growth testing was provided by a small water reservoir placed closed to the arrays in a closed Petri dish.

Prior to SEM imaging, cantilever arrays were dried at 24°C and 35% RH for at least 24 hrs. Cantilever arrays were immersed in 5% glutaraldehyde solution (0.05 M phosphate buffer, pH 7) and incubated at room conditions with gentle shaking for 3-4 h. After fixation, excess glutaraldehyde was removed by washing in phosphate buffer (0.05 M, pH 7) for 10 min. This step was repeated 6 times. Samples were subsequently dehydrated using 10-30-50-70-90-100-100% ethanol solution (10 minutes for each step). After drying, 10 nm Pd was deposited on the samples (Cressington Sputter Coater 208 HR Watford, United Kingdom). SEM images (SEM-ULTRA, Carl Zeiss, Germany) were taken at 5 kV, with an aperture size of 30,000 μm using the lens detector.

3. Results and Discussion

Microcantilever arrays were previously used to detect the viable growth of *E. coli* [10, 12] and *Aspergillus niger* (*A. niger*) [9, 13]. In these studies individual glass microcapillaries were used to seed cells on individual agarose-functionalised cantilevers. The deposition of *A. niger* spores was performed by functionalising the cantilever surface with a uniform distribution of *Aspergillus*-specific antibodies. The seeded microbial species were mixed with a culture medium which provided essential nutrition for microbial growth. After immersion of the levers in the suspension, nutrition is stored in the porous agarose layer. During growth microbes assimilate nutrition and water from the nutritive layer and humid air. In order to compare the coating efficiency of ink-jet printing to that of the previously used capillary coating method, two cantilever arrays were prepared for growth testing (Figure 2). Agarose-coated cantilevers were immersed to full, half, and 1/5 of their lengths in glass capillaries filled with *E. coli* suspension for 6, 4, and 2 minutes, respectively, (Figure 2(a)). On a second agarose-coated array the same *E. coli* suspension was deposited by spotting at different points along the cantilever. At each position, 10 drops of cell suspension were dispensed with high accuracy (Figure 2(b)). SEM images of *E. coli* colonies were taken after 24 h growth. As a control, a second set of arrays were prepared as described above; however, they were not incubated at optimal growth conditions (non growth controls). Capillary coating with these relatively large

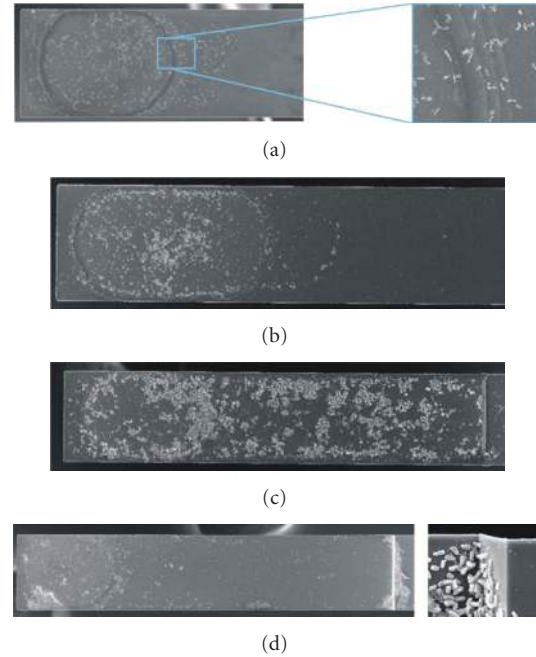


FIGURE 4: SEM images of cantilevers demonstrating accuracy of drop positioning and cell distribution after 24 h incubation at growth conditions. The width of cantilevers is 100 μm in each case. Ten drops of cell suspension were deposited at the front end of the cantilevers. (a) Imprints of series of droplets on agarose-coated cantilever, after growth of *P. aeruginosa*. (b) Ten perfectly spotted drops of *S. aureus* (2.92×10^8 cells/mL, 10% LB) cells on an agarose-coated cantilever. One spot was deliberately deposited off-position. (c) *S. aureus* (3.28×10^8 cells/mL, LB) growth on cantilever surface. (d) *P. aeruginosa* (1.84×10^8 cells/mL, 10% LB) growth on cantilever surface. Cells are growing over the clamping point of the cantilever.

structures (2 μm) results in the accumulation of cells at the capillary open end. This can be explained by the flow of cells with the liquid and evaporation at the front of the tube (Figure 2(c)). Compared to capillary functionalisation, the localised deposition of cells can be easily controlled by ink-jet printing. The sharp edge of the drop boundary visible on the SEM image (Figure 2(b)) shows that the positional accuracy of the delivered droplet is highly reproducible. Deposition of 180 droplets with 3 different dilutions of microbe suspension shows that the average droplet size and the average cell number per droplet correlate with the dilution of the samples (Table 1). There was no increase in the cell number per drop which indicates that cells are not sedimenting in the nozzle during deposition. The optimal cell number to avoid nozzle clogging in the case of bacteria is under $5-6 \times 10^8$ cfu and under $1.2-1.4 \times 10^6$ cfu in the case of *C. albicans*. It is important to mention that before glutaraldehyde fixation of samples, salt and medium residues covered the cantilevers indicating good diffusion of the nutrition along the full length of the lever. The washing and dehydration steps after glutaraldehyde fixation were sufficient to remove these residues without the removal of the deposited cells (Figure 3). We postulate that the thin liquid layer present at >90% relative humidity may act to keep the nutrition

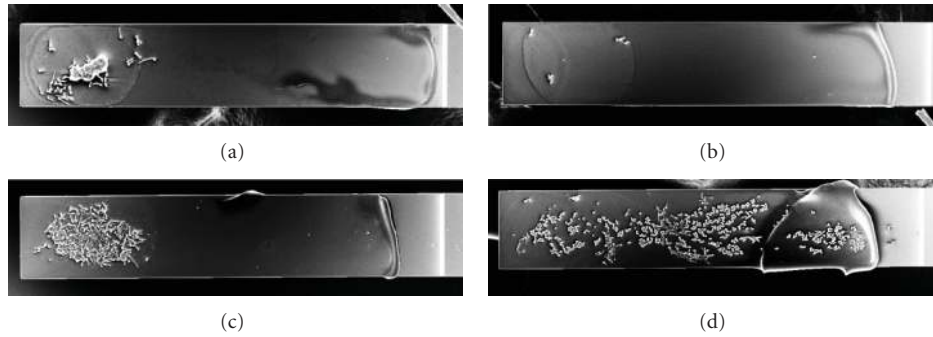


FIGURE 5: SEM images of cantilevers showing *C. albicans* propagation on the surface after 24 h incubation at growth conditions. The width of cantilevers is $100\ \mu\text{m}$ in each case. Ten drops of cell suspensions were deposited at the front end of the cantilevers. (a, c) *C. albicans* (1.36×10^6 cells/mL, 10% ME) before growth (a) after growth (c). (b, d): *C. albicans* (10^6 cells/mL, ME) before growth (b) after growth (d).

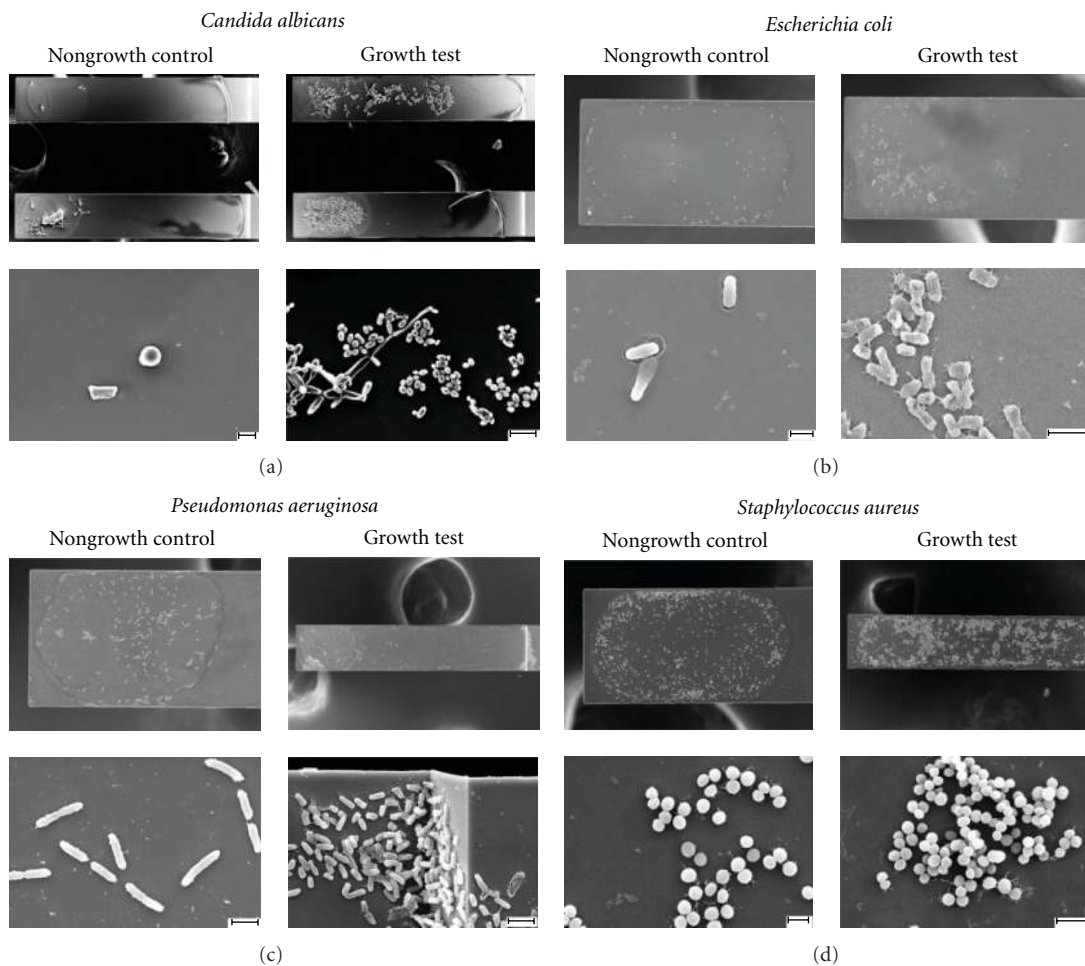


FIGURE 6: SEM images of growing microbe cultures on agarose-coated cantilevers. The width of cantilevers is $100\ \mu\text{m}$. In each section, the pictures on the left show the surface of the cantilevers dried at room conditions after ink-jet spotting. The pictures on the right show cantilevers after 24 h incubation at growth conditions. Ten droplets were deposited on the front end of each cantilever. (a) *C. albicans* suspensions deposited on the front end of two cantilevers (top: 1.36×10^6 cells/mL, 10% ME, bottom: 10^6 cells/mL, ME). Scale bars $10\ \mu\text{m}$. *C. albicans* grow pseudohypha on the surface (bottom right) (b) *E. coli* suspension (3.96×10^8 cells/mL, 10% LB). Scale bars: bottom left $1\ \mu\text{m}$, bottom right $2\ \mu\text{m}$. (c) *P. aeruginosa* (1.84×10^8 cells/mL, 10% LB). Scale bars $2\ \mu\text{m}$; (d) *S. aureus* suspension (2.92×10^8 cells/mL, 10% LB). Scale bars: bottom left $1\ \mu\text{m}$, bottom right $2\ \mu\text{m}$.

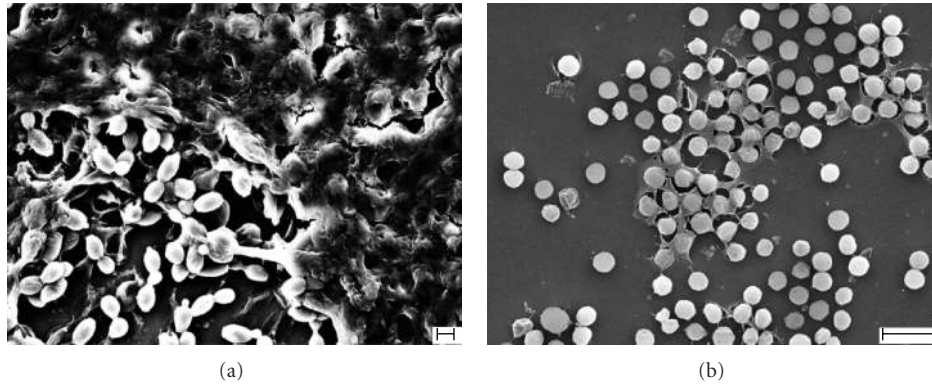


FIGURE 7: SEM images of biofilm-like structures of *C. albicans* (a) and *S. aureus* (b) on a cantilever surface. *C. albicans* cells were deposited in 10% ME medium (10^6 cells/mL) and incubated at growth conditions for 24 h. *S. aureus* cells were deposited in 10% LB medium (2.92×10^8 cells/mL) and incubated at growth conditions for 24 h.

in solution on the cantilever surface which aids in this distribution. Therefore, in an actual growth measurement, at high humidity, no salt-carbohydrate-protein crystal layer is formed which would have an effect on the mechanical properties of these sensors and the growth characteristics of the seeded microorganisms. This provides an opportunity for initial cells to spread along the surface even if nutrition is originally available only at the spotting site at the beginning of the experiment. A similar distribution of nutrition is not seen on the non-growth control cantilever which was not incubated at elevated humidity levels. In some experiments Figures 4(c) and 4(d), cells were observed outside the spotting area after growth of the culture which cannot be explained by an off-position droplet deposited during ink-jet printing (Figure 4(b)). Concentric imprints caused by individual droplets on the agarose layer are often visible on SEM images showing an accurate centre position of the droplets (Figure 4(a)). The tested *C. albicans* (Figure 5) or *S. aureus* (Figure 4(c)) cannot actively move away from their original position while *E. coli* or *P. aeruginosa* (Figure 4(d)) are motile bacteria enabling cell propagation especially if a thin liquid layer is available. This propagation is more likely if 100% culturing medium is used for microbe deposition instead of with diluted medium (10% LB or 10% ME). Dilution of the growth medium decreased the growth rate of microbes. In the case of *C. albicans*, the use of less cells per droplet (5–10 cells/droplet) in full medium still resulted in the complete coverage of the cantilever surface with cells (Figure 5(d)) when compared to the non-growth control (Figure 5(b)). Spotting 50–100 cells in diluted medium (Figures 5(a), 5(c)) did not allow the organism to grow out of their initial spotted position. In several experiments, four microbial strains were tested in terms of viability after ink-jet printing and grown on the surfaces of microcantilevers. Each array was prepared twice, and one of each was used as a non-growth control. SEM images of these experiments show the viability and multiplication of the tested organisms (Figure 6). In each case, elevated cell numbers can be observed on the arrays exposed to growth conditions (high

humidity and elevated temperature). *Candida albicans* is a yeast form microfungi with typical cell dimensions of 4–5 μm in diameter. At certain conditions, mostly related to limited nutrition, they are able to grow pseudohyphae where the cells have not separated after cell division. This morphological switch is often studied as it has a major role in *C. albicans* pathogenicity [14]. In our tests, *C. albicans* cells were seeded in the budding-yeast form in 100% ME suspension and also 10% ME suspension. Figure 6(a) shows that in the suspension with full medium some cells are developing filamentous structures (pseudohyphae) but also budding-yeast form can also be observed. It is likely that in this special microenvironment the pseudohyphae play a part in the spread of *Candida* along the cantilevers. All these processes resulting in the colonisation of the surface might cause changes in the cantilevers mechanical properties, and; hence, influence growth detection measurements which are originally based on the detection of a mass increase on the cantilever surface. For some biosensing techniques, high salt or sugar content of microbial culturing medium can disturb the measurement although decreasing the available nutrients in the environment can cause changes in growth rate or morphology of the cells. In other experiments *C. albicans* (Figure 7(a)) and *S. aureus* (Figure 7(b)) cells were deposited on agarose-coated cantilevers with diluted medium. We found that decreasing nutrition in the used suspension results in a decrease in viability and also altered the morphology of the cells. Cells started to accumulate extracellular biomaterial which resembled biofilm formation (Figure 7). Bacteria and *Candida* colonies usually form biofilms on various surfaces including silicon in order to help the bacterial population to survive in certain environments. However, further investigation has to be done to understand this process in the case of microcantilevers. It is expected that biofilm formation will influence the spring constant (k) of a cantilever. It is also important to mention that the use of deionised water for cell deposition resulted in the centering of bacterial cells with respect to droplet positions, while the use of more viscous suspensions, such as culture medium, resulted in the accumulation of cells around

the rim of the liquid droplet, thus, allowing for high accuracy when positioning microorganisms within a droplet (data not shown).

4. Conclusion

It was successfully proved in our experiments that different types of microbial cells are able to be deposited on microcantilever surfaces while retaining their viability. Ink-jet spotting was used for cantilever functionalisation with viable cells, thus, revealing its potential as a superior alternative for sensor applications and real-time growth measurements. There is no need for specific antibodies facilitating tight binding during deposition of cells. In the case of motile microorganisms the fluid film allows cells to be freely distributed, and, in these cases the introduction of antibodies could keep the species in place. Compared to the previously reported capillary-coating technique ink-jet printing has been shown to be a more accurate way to position cells, and hence, to optimise the detection of mass changes during microcantilever-based growth detection.

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