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IN FOCUS: NANOMEDICINE - ARTICLE

Nano-Mechanical Transduction of Polymer Micro-Cantilevers to Detect Bio-Molecular Interactions

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Abstract Using variothermal polymer micro-injection molding, disposable arrays of eight polymer micro-cantilevers each 500 μm long, 100 μm wide and 25 μm thick were fabricated. The present study took advantage of an easy flow grade polypropylene. After gold coating for optical read-out and asymmetrical sensitization, the arrays were introduced into the Cantisens® Research system to perform mechanical and functional testing. We demonstrate that polypropylene cantilevers can be used as biosensors for medical purposes in the same manner as the established silicon ones to detect single-stranded DNA sequences and metal ions in real-time. A differential signal of 7 nm was detected for the hybridization of 1 μM complementary DNA sequences. For 100 nM copper ions the differential signal was found to be (36 ± 5) nm. Nano-mechanical sensing of medically relevant, nanometer-size species is essential for fast and efficient diagnosis.

Abbreviations

MEMS	Micro-electro-mechanical systems
NEMS	Nano-electro-mechanical systems
μC	Micro-cantilever
PP	Polypropylene
μIM	Micro-injection molding
ssDNA	Single-stranded DNA
GSH	Glutathione
MCH	Mercaptohexanol
SAM	Self-assembled monolayer

1 Introduction

Medical diagnostics is a vital part of routine clinical practice. Monitoring human biological systems at the molecular level using nanodevices and nanostructures brings nanotechnology closer to nanomedicine. For better diagnosis, nanometer-size species, i.e. biomolecules, have to be recognized with high reliability, high sensitivity and selectivity within short periods of time. Size reduction of diagnostic devices decreases the amount of analyte, thereby leading to faster analysis. Therefore, micro and nano electro-mechanical systems (MEMS and NEMS) have attracted much interest for biomedical applications, often termed as nanoanalytics in the field of nanomedicine.

Micro-fabricated cantilevers and cantilever arrays belong to the promising biosensors under MEMS. For example, their high sensitivity and selectivity was demonstrated analyzing DNA sequences [1–3]. They can also operate as artificial olfactory and gustatory organs with sound performance [4–6]. The static working principle of

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such nanomechanical transducers comprises the conversion of the (bio) chemical reaction of interest on the active side of the cantilever into surface stress thus leading to the bending of the cantilever [7]. The optical readout ensures the detection of this bending with nanometer precision. In order to achieve selectivity, well-selected recognition elements are integrated on the active side of the micro-cantilever (μC). In general, silicon technology has been applied to prepare these μC arrays, which are commonly designed for single usage. Multiple uses for clinical applications are in most cases impossible. Therefore, such sensor systems are relatively expensive. Replacing the silicon-based μC arrays by means of low-cost injection-molded arrays from appropriate polymers is crucial not only to lower the costs but also to open up a broad spectrum of applications, for example in intensive care units [6, 8].

The substitution of silicon by a polymer such as polypropylene (PP), seems to be reasonable since the sensitivity of the cantilever sensor (deflection Δ_z, differential surface stress $\Delta\sigma_{\text{surface}}$) depends on the mechanical parameters (Young's modulus E and Poisson's ratio ν) and the geometry (length L, thickness t) as demonstrated more than a century ago [9].

$$\Delta_z = \frac{3(1-\nu)L^2}{Et^2}(\Delta\sigma_{\text{surface}})$$

Simple estimations show that typical polymer cantilevers can retain the sensitivity of silicon cantilevers despite a factor of 5–10 increase in their thickness. Therefore, micro-injection molding (μIM) can be applied to fabricate low-cost disposable μC arrays [10]. μIM allows an easy switch to comparable alternative polymers and for surface micro-structuring by means of methods such as embossing. Compared to the distinct single crystalline Si cantilevers, the polymer μCs within an array exhibit slight variation in geometry. For that reason, the polymer μC arrays have to be tested for their suitability in biomedicine. This includes the direct comparison with the well established Si-cantilever experiments [11–13].

Rather simple examples are the hybridization of DNA fragments with single-stranded DNA (ssDNA) or oligonucleotide that are covalently immobilized on a gold-coated cantilever taking advantage of thiol chemistry [1, 2, 11–14].

The metal ion sensing applications of the polymer μCs was studied using glutathione (GSH) monolayer's affinity for copper ions. Copper plays an essential role in human physiology. Copper ions are considered as multifunctional participating in a broad spectrum of intracellular processes under normal and pathologic conditions. The intracellular concentration of copper is tightly controlled. Exchangeable copper in cytosol is bound to small protein carriers called metallochaperones like GSH. GSH, a tripeptide from glycine, cysteine and glutamate, is the most abundant non-protein thiol-bearing molecule of mammalian cells and is involved in many physiological processes [15–17]. GSH is known to interact with ions and heavy metals, and is capable to organize on gold surfaces as self assembled monolayers (SAMs) [18]. SAMs serve as sensitive sensing layer for cantilever based sensors. Gold coating can be performed for silicon and polymer in similar manner. In both cases, experience exists as gold has been deposited as reflection layer for optical read-out of cantilever deflection. Nevertheless, it has to be demonstrated that polymer cantilever sensors reach the desired selectivity, sensitivity, and reliability for the detection of relevant bio-molecules and metal ions.

2 Materials and Methods

2.1 Cantilever Fabrication

Arrays of eight polymeric μCs (see Fig. 1) were fabricated using variothermal μIM. The setup was similar as described in [10], consisting of a metal (Polmax Uddeholm) mold insert (90 × 100 mm^2) mounted in the clamping unit with millimeter-size cavities, which was closed by a flat counterpart (mirror unit) containing the injection gate. The molding tool was installed in an Arburg 320 Allrounder (Arburg, Lossburg, Germany) with a maximum clamping force of 600 kN. The mirror unit of the molding tool, a polished steel surface ensured an optically flat and smooth surface. On the tool side 16 branches of 2.5 mm wide and 60 mm long channels with semi-cylindrical shape were ordered around the central point opposite to the injection gate, all ending in 3.5 × 2.5 × 0.5 mm^3 cavities representing the μC array holder cavities (Fig. 1d).

The eight nominally 25 μm-deep, 500 μm-long and 100 μm-wide μC mold cavities were laser ablated between the holder cavity and millimeter-long, 20 × 20 μm^2-wide venting channels. Due to the laser ablation process, the μC mold cavities are not rectangular. The cross-section exhibits a trapezoid form (Fig. 1b), with a widening of about 10 μm on each side from the laser-ablated bottom of the cavity to the top, which during injection molding is formed by the mirror side of the molding tool. Due to fabrication tolerances, the widths of the individual μC mold cavities vary from 83 to 88 μm, leading to variations in the mechanical behavior. For variothermal heating, heat cartridges were placed directly below and above the μC mold cavities, i.e. on both the tool and the mirror sides, thus enabling localized heating above the tool temperature. As polymer, an easy flow grade polypropylene (PP Metocene HM 648T, LyondellBasell, Bayreuth, Germany) served for the studies presented in this paper. The ejector pins in each channel ensured easy demolding. The parts were manually

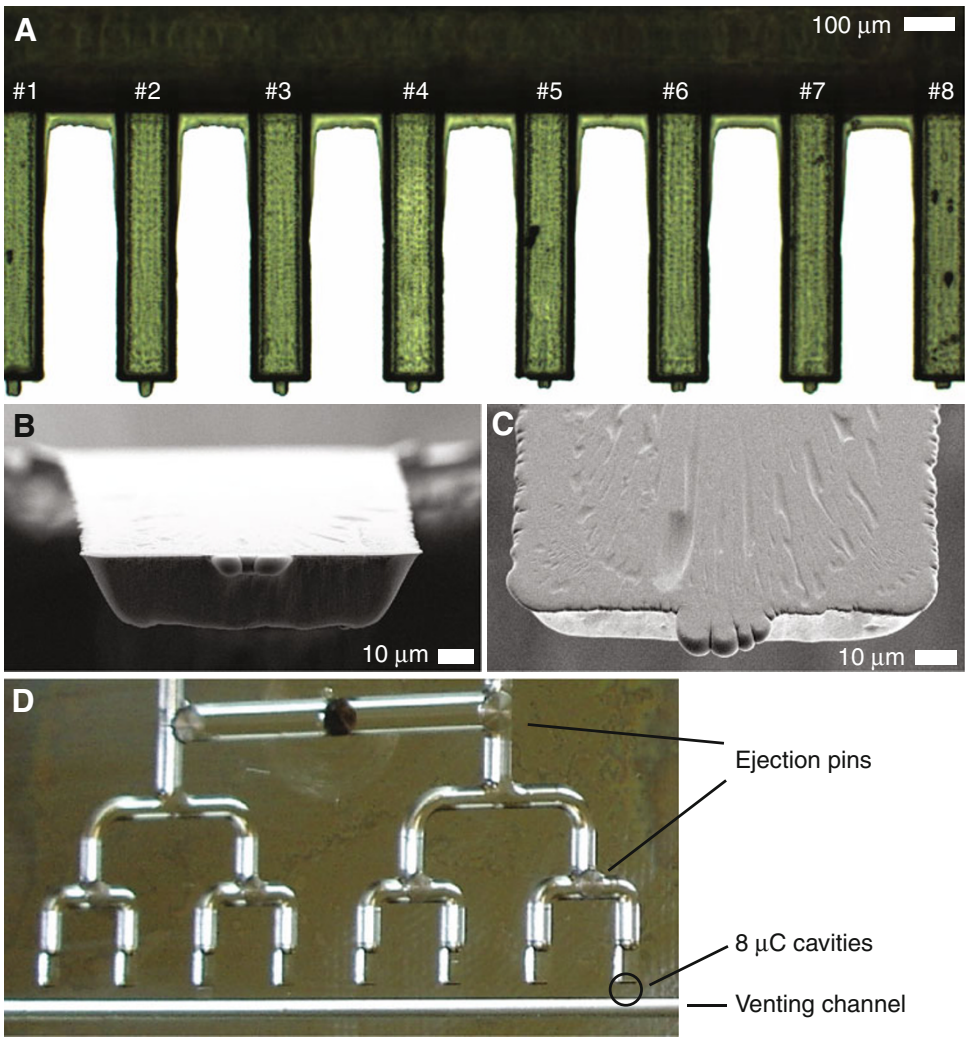

Fig. 1 Optical micrograph of a variothermal micro-injection molded PP µC array, each cantilever is 500 µm long, 100 µm wide and 25 µm thin (**a**), SEM micrograph of the trapezoidal form of the PP µC (**b**), SEM micrograph of the surface of the PP µC (**c**), photograph showing a part of the mold insert (**d**)

removed from the mold, i.e. no mold release agent was applied. The process parameters were similar to the one's given in Ref. [10], i.e. melt temperature 200°C, tool temperature 40°C and injection speed 9 cm^3/s. Using variothermal µIM process parameters (increase of 30 K over tool temperature at the beginning of each molding cycle), the complete filling of the high-aspect-ratio microcavities was achieved, as demonstrated by the microscopy images in Fig. 1. The µC arrays were produced in batches of 20 arrays. Further details on the fabrication of PP and other polymeric µCs will be given in a forthcoming publication in an engineering journal.

2.2 Cantilever Functionalization

To functionalize cantilevers with receptor molecules and also to ensure sufficient reflectivity of the laser signal, the PP µCs were coated on the replicated mirror side with a 4 nm-thin chromium film (Umicore; Code 0702723, Cr 99.99%, Flakes 2.8–4.7 mm) followed by a 20 nm-thin gold film (Umicore; Code P0481088, Au 99.99%, 2 mm wire) using a thermal evaporator (Balzers BAE 250, Balzers, Liechtenstein). The film thickness was controlled by means of the quartz crystal microbalance integrated in the evaporation system.

Prior to functionalization, the µCs were treated in a UV/Ozone cleaner (UV Clean Model 13550, Boekel Scientific, Feasterville, PA). This procedure yields a well-defined gold surface, which is crucial for the thiol immobilization. To avoid chemical degradation of the PP, the UV-ozone treatment was limited to a period of 20 min.

The µCs were functionalized by immersing them for a time period of 30 min in glass capillaries filled with experiment specific functionalization solution using the Cantisens® FU-401 unit (Concentris GmbH, Basel, Switzerland). In order to prevent any evaporation of the experiment specific functionalization solution, the procedure was carried out in humidified atmosphere.

For the copper ion sensing experiments, µCs #2, #4, #6, #8 were coated with glutathione (GSH, Sigma Aldrich, Buchs, Switzerland) in Cu-functionalization buffer solution

(100 mM NaCl, 10 mM Tris) [11], while the other four bare μCs served as references.

For the DNA hybridization experiments, μCs #1, #2, #5, #6 were functionalized with ssDNA thiol-Nl4-3 sequence (Microsynth AG, Balgach, Switzerland), and μCs #3, #4, #7, #8 with ssDNA thiol-Sf162 (Microsynth AG, Balgach, Switzerland) in DNA-functionalization buffer solution (200 nM NaCl, 20 mM Tris), as described in detail earlier [11]. In short, μCs #1, #2, #5 and #6 were functionalized first, followed by μCs #3, #4, #7, and #8 according to the geometry of the functionalization unit generally applied for Si cantilevers. The human immunodeficiency virus type 1 (HIV-1) strain thiol-Sf162 (CAT ACA ACA GGA AGA ATA ATA GGA G) and thiol-Nl4-3 (GTT ACA ATA GGA AAA ATA GGA A) were used as the sensing sequences.

2.3 Mechanical Characterization of Cantilevers

The gold-coated PP μCs were introduced into the water-filled measurement cell maintained at a temperature of 25°C. Using the optional liquid handling system from the integrated temperature control in the Cantisens® system, a temperature profile program under static conditions was setup. The temperature was increased with a rate of 0.2 K/s from 25 to 30°C, then holding constant for 240 s and then decreased back to 25°C with a rate of −0.2 K/s. Subsequently, such a cycle was performed using a temperature difference of 10 K, i.e. from 25 to 35°C and back to 25°C (see Fig. 2).

2.4 Monitoring Surface Stress During Thiol Adsorption

The characterization of thiol functionalization on gold-coated PP μCs was conducted applying a constant flow of 0.42 μL/s at a temperature of 25°C. 100 μL of 0.1 mM mercaptohexanol (MCH, Sigma Aldrich, Buchs, Switzerland) diluted in water was injected into the pumping loop and the cantilever bending during the real-time chemisorption of thiol on the gold was recorded. This is a test for the mechanical behaviour of the cantilevers when a chemical reaction takes place at the surface.

2.5 Analytical Procedure

The measurements were done using the Cantisens® Research platform, which allows for real-time experiments. The experiments (copper sensing and DNA hybridization) were conducted with a constant flow (0.42 μL/s) at a temperature of 30°C. For MCH treatment, 0.1 mM of MCH diluted in the experiment specific running buffer solution, i.e. Cu-running buffer and DNA-running buffer, was prepared and introduced into the measurement cell. The measurement cell and the connecting tubes of the pumping loop including valves were cleaned before each injection.

The glutathione-functionalized μCs were rinsed for several minutes in the Cu-running buffer (100 mM NaCl, 0.5 mM EDTA, 10 mM Tris, 0.005% Tween 20, pH 7.5) to remove excess adsorbed glutathione. The sample (analyte) solution was prepared dissolving copper chloride ($CuCl_2$, Sigma Aldrich, Buchs, Switzerland) in the Tris buffer (100 mM NaCl, 10 mM Tris, 0.005% Tween 20, pH 7.5) to a final concentration of 100 nM.

The sample (analyte) solution used in the DNA hybridization experiment was 1 μM complementary Sf162 diluted in the DNA-running buffer (1 M NaCl, 20 mM Tris pH 7.2, 0.005% Tween). The μCs were regenerated after each experiment washing them with 30% urea (Sigma Aldrich, Buchs, Switzerland) solution, which completely removes hybridized complementary ssDNA [1].

3 Results

3.1 Mechanical Characterization

For applying cantilevers in sensing applications, it is important that the cantilevers bend homogenously, thereby requiring their characterization. The mechanical behavior of the μCs was tested by thermally induced bending of the asymmetric cantilever, often termed heat test. The heat tests included a temperature cycle from 25 to 30°C and 25 to 35°C as described above. Due to the difference in the thermal expansion of the 24-nm thin metal layer and the 25-μm thick PP layer, compressive stress is generated resulting in a deflection. The deflection from the central four μCs (#3, #4, #5, #6) is almost twice that of the outer four μCs (#1, #2, #7, #8). For a temperature difference of 5 and 10 K, the maximum deflection for the central PP μCs in water corresponds to (365 ± 20) nm and (800 ± 50) nm, respectively (cp. Fig. 2).

3.2 Thiol Adsorption

Creation of homogeneous monolayers on cantilever surfaces forms the basis of most sensing experiments. The thiol compounds have a high affinity for the gold-coated surface of the μCs and bind to the gold forming a densely packed SAM. The deflection caused by injection of 0.1 mM MCH is shown in Fig. 3. μC #8 was omitted due to its insufficient reflectivity for read-out. Surface stress generated during the growth of the self-assembled thiol monolayer led to significant cantilever bending. A maximum deflection of (110 ± 10) nm was recorded in real-time for the μCs (with exception of μC #5). Using the Stoney formula [9, 19], the differential surface stress was

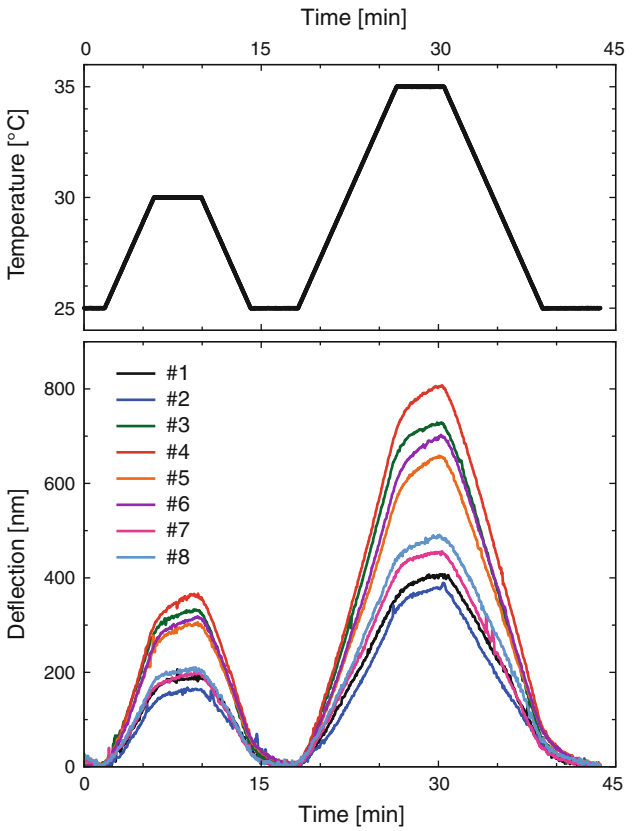

Fig. 2 The graph on the *top* shows the temperature cycle and the graph *below* shows the response of the variothermal molded PP μCs

calculated to a value of (0.28 ± 0.03) N/m^{-1}. The PP μCs bend continuously until saturation lasting for a minute. The SAM formation monitored in real-time is a fast process as seen in Fig. 3 which can be interesting for biomedical applications.

3.3 Copper Sensing

When copper ions are injected in the measurement chamber, the glutathione-functionalized cantilevers bind the divalent copper ion and generate a related deflection signal. The injection of 100 μL of 100 nM $CuCl_2$ causes a shift in the differential signal of (36 ± 5) nm corresponding to a differential surface stress of (0.090 ± 0.002) N/m^{-1} as clearly shown by the data in Fig. 4. The decrease in deflection after 5 min is due to the instability of the experimental setup and lies within the error bars. Figure 4a shows the deflection of the individual μCs including the reference μCs (#2, #4, #6, #8) and signal μCs (#1, #3, #5, #7).

3.4 Detection of DNA Hybridization

DNA hybridization is caused by the complementary interaction between probe DNA and sample DNA [14]. The difference between the average signal μCs (#3, #4, #7,

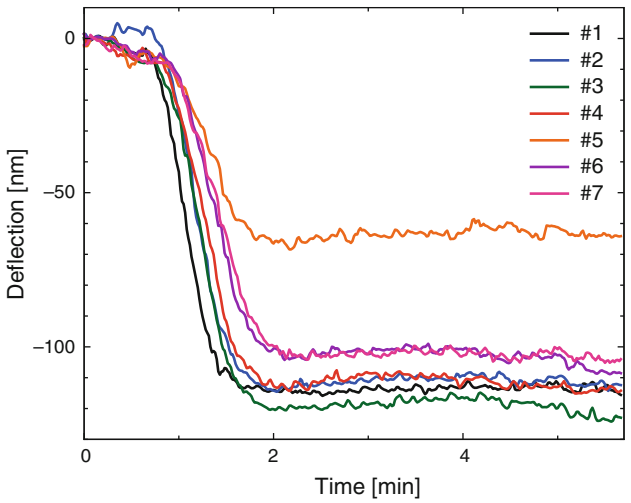

Fig. 3 Surface stress generated during the formation of MCH SAM on the gold layer on the active side of the PP μCs

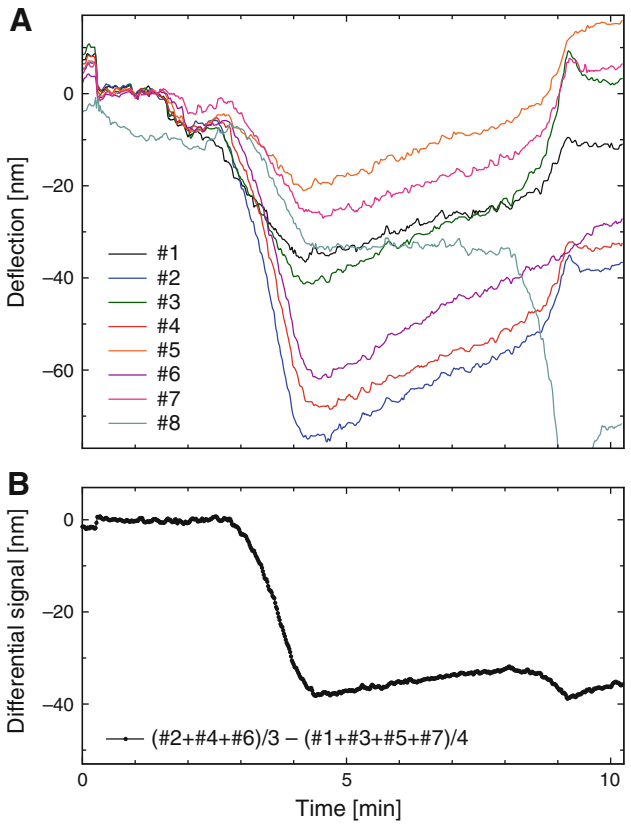

Fig. 4 Deflection of the reference μCs and GSH-functionalized PP μCs upon $CuCl_2$ injection (**a**), Differential signal from Cu^{2+} ions binding to GSH-functionalized PP μCs (**b**)

#8) deflection and the average reference μCs (#1, #2, #5, #6) deflection is shown in Fig. 5. The differential signal is generally required because of thermal drifts and unspecific interactions. The first injection of 100 μL of 1 μM complementary Sf162 sequence gives a signal of 7 nm

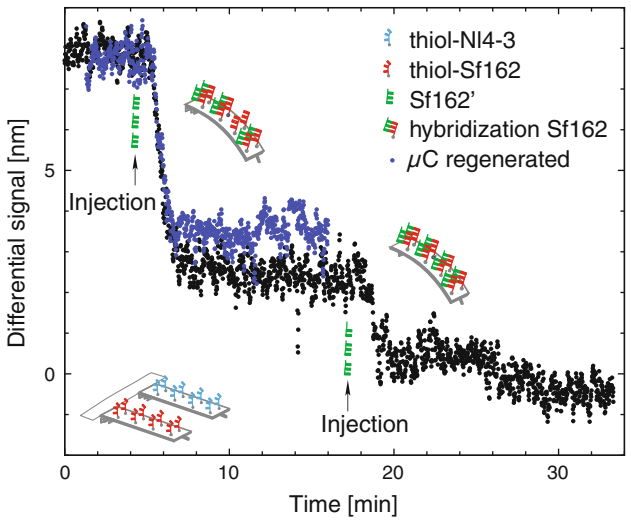

Fig. 5 Differential deflection upon hybridization of the complementary ssDNA sequence with the thiol-ssDNA sequence attached on the gold

($\Delta\sigma_{surface} = 0.02$ N/m^{-1}), which is comparable to the signals achieved with Si cantilevers. The differential deflection of 7 nm corresponds to 25% of differential deflection obtained with the 1 μm-thin silicon cantilevers [11, 14]. A second injection of the same complementary sequence was a control for measuring the saturation level of the first injection and led to an additional 1.5 nm differential signal. After regeneration, the DNA experiments give the same signal within the error bars. Figure 5 also shows the differential deflection signal of DNA hybridisation after regeneration. The result demonstrates the application of cantilever sensors for the detection of ssDNA sequences via their hybridisation with sensing ssDNA immobilised on the cantilever. It is important that the reference cantilevers are as similar to the sensing cantilever as possible. Note that a bare cantilever cannot be used, as the sample DNA interacts with the bare cantilever leading to a bending signal. Therefore, similar DNA sequences have to be used as reference. The HIV-1 sequences Nl4-3 and Sf162 are 70–80% homologous to each other. Hence Nl4-3 sequence was used as the reference. Using homologous sequences, the specificity of the DNA hybridization can be detected and distinguished by the cantilever sensors.

4 Discussion

Complete filling of the 25 μm thick, 500 μm long cavities using variothermal μIM was possible using the easy flow grade PP. The 25 μm-thick PP μCs are far too thick compared to the estimated 5–10 μm thickness needed for direct comparison to the standard 1 μm-thick Si μC. According to the Stoney formula, their mechanical behavior would correspond to 4 μm thin Si μCs. However, our results show that sufficient selectivity can be achieved with the 25 μm-thick PP μC, too. With the current setup and parameters, μCs much thinner than 25 μm are difficult to achieve, particularly if tight homogeneity aspects have to be met. However, better results can be expected with optimized temperature gradients within the mold and possible thermal treatment procedures after appropriate demolding. The molding process can be further tuned to incorporate other biocompatible polymers, however, only if similar flow properties can be met. The current aim is rather to improve sensitivity by implementing surface structures than by further reducing the cantilever thickness. This concept was demonstrated in Ref. [10], but will be investigated in more detail in future research. Further advances in polymer μCs can be promising in developing sensors with suitable sensitivity and selectivity.

In the heat tests, upon temperature change, the biphasic μC deflects due to the bending moment generated by the different thermal expansions of the two materials [11, 20]. We have shown that PP μCs with a thin gold film on one side undergo measurable bending in response to temperature changes. The deflection signal with the variothermal injection molded μCs is larger as compared to the ones molded in a non-variothermal mode [10]. These gold-coated μCs have the potential to distinguish effects of temperature and can be used in applications requiring high precision thermal sensitivity. The difference in deflection of the outer cantilevers to the central ones is a severe drawback. It might be significantly reduced identifying suitable temperature gradients within the mold and applying appropriate demolding procedures. Furthermore, the fixation mechanism of the cantilever array might lead to a tilt that stiffens the outer cantilevers. Therefore the fixation should be optimized. It must be noted that the deflections shown in the heat tests are huge as compared to the deflections observed in biosensing experiments. Mechanical characterization of each disposable PP μC array is necessary before meaningful experiments can be carried out.

The formation of SAMs is of great interest in the field of surface science. SAMs of highly ordered and oriented alkane thiolates provide a prominent, flexible, and convenient way to generate well-defined organic surfaces with useful and highly alterable chemical functionalities displayed at the exposed interface [21]. It has been shown that the deflection during the chemisorption of thiols is due to the surface stress and the thermal effects involved in the exothermic thiol SAM formation are negligible [19]. A clean and smooth gold surface is a pre-requisite for uniform thiol adsorption. The large deviation in the deflection of μC #5, for example resulted from

inappropriate gold coating (see Fig. 1a). The degradation of the PP μC surface and the gold coating during the UV/ozone treatment cannot be ruled out either. The thiol adsorption measurements elucidate the necessity of calibration before reproducible experiments can be performed, reliably. The sensing applications of μCs including artificial noses needs calibration even for silicon.

The ion sensing properties of the GSH SAMs are demonstrated with the copper sensing experiments. Sensing properties of GSH SAMs are promising and can be extended to further applications such as metal concentration after chemotherapies.

Increased levels of copper in cerebrospinal fluid in patients with Alzheimer's disease have been found [22]. Further experimental studies would aid the discovery of effective Cu biomarkers and the generation of new options for early intervention in copper-related health disorders.

Fritz et al. [1] already showed that the hybridization of complementary DNA is measurable giving rise to a surface stress change of 1 mN/m and a mismatch between two 12-mer oligonucleotides is clearly detectable. The detection of ssDNA hybridization was chosen as a model experiment to demonstrate the effectiveness of PP μCs as biosensors. MCH served as a blocking molecule between the immobilized thiolated ssDNA molecules to prevent unspecific interaction of complementary DNA with the gold surface. Two 18-mer oligonucleotides, which are 70–80% homologous, were used in our hybridization experiments as compared to the 12-mer oligonucleotides reported earlier [1, 2]. For optimal distinction of hybridization between the 70 and 80% homologous strands, the temperature of the experiments were elevated to 30°C. The higher temperature helps to gain specificity of DNA hybridization with longer nucleotides. The response of PP μCs during the hybridization of complementary ssDNA with the probe ssDNA is shown. The bending direction of the μCs (bend up or down) during hybridization is consistent with the Si μCs. However, the magnitude of the displacement is four times smaller than for the Si μCs which corresponds to their higher stiffness coming from a significantly larger thickness of the polymer μCs. The sensitivity of the cantilever sensing is determined by the number of the binding events that give rise to the surface stress. With 18-mer oligonucleotides, the number of DNA molecules per cantilever is smaller than 10^{10} probes per cantilever as mentioned earlier [2]. The reduced number of DNA molecules per cantilever can lead to a reduction in signal but increase in sensitivity. The cantilever surface with ssDNA probes can open options for detection of target genes or antigens. It can be further extended to measure single nucleotide polymorphisms with μC sensors in a fast and easy way.

5 Conclusions

Within many areas of medicine, there has been a constant need for technical developments and sophisticated methods. The development of a simple, portable, clinical diagnostic device that can perform a comprehensive range of tests, inexpensively, and give results within a few minutes has been a dream of biosensing research groups for many years. Such a device will have the capability to make diagnoses, monitor critical clinical indicators and tailor treatments, accordingly. Since results are available on site, remedial action can be initiated. With the polymer microcantilevers shown here, a first step towards this goal is demonstrated. In comparison to the silicon cantilevers used until now, comparable results can be achieved. The advantage of our technique is that a range of polymers can be used for injection molding thereby adapting the mechanical properties of the cantilever sensor to the desired application. As an added advantage, the polymer μCs can easily be surface patterned [10] to increase sensitivity or to enhance specific interaction with cells.

Cantilevers sense surface-associated processes like conformational changes or molecular interactions. The rapid, real-time detection of interactions/biomolecules is clearly an advantage of the label-free biosensor, allowing further applications in basic research and sensing [11, 23, 24]. μC biosensors hold promising perspective in the applications of medical diagnosis through the development of miniaturized, low-cost sensor platforms. The μIM process provides a fast, inexpensive and reliable method to obtain arrays of μCs [10]. With eight cantilevers in an array, eight different species or bio-molecular interactions can be detected with no extra label or tag. PP μC arrays have proven their selectivity to detect DNA sequences and distinguish homologous sequences. Currently, the detection limit lies in micro-molar concentrations, but can be significantly lowered to nano-molar concentrations reducing cantilever thickness. In future cantilever sensors with improved design, tooling and process parameters, can lead to medical diagnostic devices that are portable and inexpensive, useful in the detection and diagnosis of human diseases.

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