Tunable and reversible gelatin-based bonding for microfluidics: manufacture of cell culture chips

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Abstract

Microfluidic cell culture is widely used to develop biochips and biosensors, culturing and experimenting with cells at the microscale. However, only a very small subset of the existing polymers is currently used in microfluidics. This is mostly due to limitations in reversibility and gas-permeability on the sealant. Hence, the development of a novel bonding technique can enable new applications and uses of plastics in microfluidic cell culture, complementing the omnipresent polydimethylsiloxane (PDMS) for critical applications where harder or non-porous materials are required. The present paper describes a reversible gelatin-based room-temperature method for bonding separate substrates, which enables the sealing of commonly used materials in microfluidics such as thermoplastics, but also elastomers and photopolymers. For most materials, the bonding chip resisted to at least 0.1 MPa. To show the versatility of the described method we bonded microchannels of different sizes, up to 200 µm, and round microstructures. The applicability to cell culture was investigated by culturing colorectal cancer HT-29 cells within the chip. Finally, the cells viability was analyzed by in situ live/dead fluorescence staining. Advantageously, the proposed bonding process is reversible and make possible to tune the permeability of the gelatin layer integrated on chip. This room-temperature bonding method is highly efficient for cell culture in plastic chips, potentially opening new routes for the development of innovative BioMEMS devices.

Keywords: Bonding, Microfluidics Gelatin, Cell Culture, Biosensor

1 Introduction

Since its beginnings in microelectronics and analytical chemistry, microfluidics has become increasingly unavoidable in different fields such as: biophysics, biochemistry, biotechnology and biomedicine [1]. For instance, ~~O~~one of the key benefits of microfluidics in~~for basic~~ biology is the ability to finely control parameters of the cell microenvironment at microscale [2]. In this respect, ~~S~~several platforms, generally made of polydimethylsiloxane (PDMS) [3], have been ~~were~~ recently applied to~~for~~ long term cell culture and assay system compatible with different microscopes and sensors, for ~~long term cell culture,~~ high throughput time lapse cell assays/imaging and cell micromanipulations [4, 5].

The microfluidic technology overwhelmingly relies on microchannels, a set of microstructures etched or mold into a wide range of material including glass, silicon or polymer such as PDMS and poly(methyl methacrylate) (PMMA), etc. [6]

The major processes of the chip manufacturing are deeply rooted in cleanroom microfabrication ~~methods~~ (e.g. microlithography, doping, thin films, etching and bonding) [7, 8]. In addition to these methods~~tools~~, the development of bonding approaches~~methods~~ for the sealing of different substrates is obviously crucial~~extremely important~~ in the field of microfluidics and numerous methods have been therefore developed over time to address this need [9]. Sealing of microfluidic chips allows for example to: (I) confine liquids and biological samples in reduced volumes, (II) minimize contaminations and biohazards and (III) control~~minimize~~ the evaporation of samples and reagents while ensuring the exchange of oxygen and nutrients etc. ~~Nonetheless~~Unfortunately, most of the reported methods are limited~~restricted~~ to irreversible bonding [10-21] and often, when plastics ~~polymers~~ are bonded, the chips are not totally suitable for~~applicable to~~ cell culture due to the limited exchange of gas and nutrients. The irreversible bonding is a major limitation for BIOMEMS systems and organ/tissue on chip applications ~~for other two reasons:~~since it complicates off-chip characterization of the cultured biosamples, and prevents reusing successful microchannels, which exacerbates the issue of difficult access to cleanroom facilities faced by many biological research laboratories. In order to overcome these hurdles, ~~A~~alternative approaches have been prposed~~reported~~ to temporary seal~~bond~~ the microfluidic chip layers~~s~~ [22]. For example, Serra et al. recently described the use of a commercially available PCR adhesive tape, as a versatile bonding solution for microfluidic

channels patterned on~~in~~ a wide variety of materials [23]. ~~In 2010,~~ Kitamori and co-workers developed a modular microfluidic chip using a metal chip holder (screws) and surface chemistry modification, to recover the living cells cultured in the microchannel [24]. R~~More r~~ecently, we applied a magnet-integrated modular chip for 3D cell culture and post experiment recovery of the biomanufactured microtissues [25]. While such reversible sealing leads to high throughput, ease of fabrication, cost effectiveness, and ~~more~~ functional devices, its major limitation is, in some cases, the poor sealing strength and leakage [22]. ~~Moreover,~~Generally, when compared to irreversible bonding, the ~~se~~ temporary methods often show scarce~~poorer~~ reproducibility as they require manual operations such as ~~the~~ adhesive tape application, ~~and the~~ screw~~ings~~ or magnets manipulation~~integration~~.

In order to possibly solve the reproducibility issue, related to manual operations ~~need to be avoided altogether, and~~, several coating techniques based on liquid adhesive layers have been~~were~~ proposed to bond microfluidic chips [9]. One of the most widely used ~~adhesive~~ bonding technique~~s~~ is based on the application of liquid adhesives (glues) that hardens by evaporation of a solvent, by UV mediated cross-linking ~~after UV exposure~~, or by curing with ~~mixing~~ with a chemical cross-linker (i.e. catalyz~~er)ing agent~~. For example, Dupont et al. fabricated microfluidic chips having a channel layer structured by casting of Norland Optical Adhesive (NOA) 63 and sealed by a membrane of NOA 63 [26, 27]. Despite the efficiency of this method, NOA adhesive technology makes it impossible to separate the substrates. Another challenge associated with NOA is the difficulty of obtaining a uniform thickness along large surfaces in order to avoid clogging of microfluidic channels during bonding. Furthermore, the NOA substrates show a low gas permeability and therefore the applicability to microfluidic (plastic chips) cell culture is reduced. ~~Another commonly adhesive used is PDMS, proposed with a technique called ‘‘stamp and stick’’, where a thin layer of adhesive, such as an uncured PDMS or UV adhesive, is selectively transferred to the microfluidic layer using a stamping process, then bonded to a substrate [28, 29]. Similarly to NOA, the PDMS adhesive technique is not completely suitable for reversible systems, especially for fully PDMS chips. In addition, the PDMS as adhesive materials requires access to cleanroom facilities or high-end equipment and cannot be applied to assembly hydrogel layers~~.

BFurthermore, biomaterials, and particularly hydrogels, have been developed and implemented for investigating countless cellular processes towards understanding morphogenesis, aging, disease and more recently to develop 3D cell culture [30, 31]. A bonding method based on hydrogel is highly desirable in microfluidic cell culture, also for the potential assembly and development of fully-hydrogel chips for organ on chip applications.

The present – paper describes a room-temperature adhesive method based on gelatin dehydration (GEL-D) to bond two or more chip parts (i.e. layers (or slab) possibly made of different materials. It is based on the formation at the material interfaces of an integrated, biocompatible and gas-permeable thin gelatin film byusing a user-friendly spin coater or, alternatively, a modified computer cooling fan [32]. The core mechanism of the proposed method is to contact the gelatin-coated layers via a mild compression and cross-link the sandwiched film by dehydration. The gelatin dehydration was recently applied in microfabrication processes [33], but was never exploited, to the best of our knowledge, to bond microfluidic chips. To demonstrate the efficacy of our bonding method, the leakage within the fabricated chips was tested (Movie 1). In addition, the pressure resistance was measured, exceeding for some chip configuration the maximum pressure (Pmax) of our controller: 0.70 MPa. Despite the reversible nature of the gelatin dehydration, we found the chips to last up to 11 days in a cell biology incubator (cell culture conditions: 37°C, 5% CO2 and 95% relative humidity). Furthermore, the biocompatibility of the proposed method for on-chip cell culture was also evaluated by seeding HT-29 colon adenocarcinoma cells within the chips. In particular, as expected using a biocompatible gelatin, the microchannels allowed successful cell culture and fluorogenic on-chip live/dead experiments. Indeed materials transparency enables high-resolution optical microscopy observations. Finally, the possibility to grow a tumor microtissue on chip over 7 days within a chip sealed by the GEL-D method was also verified. The off-chip characterization was performed, thanks to the reversibility of bonding. Thus, the proposed reversible bonding method is compatible with the majority of common microfluidic materials (hard and flexibles) and making it extremely useful for cell culture on chips made of plastic materials.

2 Materials and methods

2.1. Materials and equipment

The PDMS prepolymer and curing agent (Sylgard 184 elastomer kit) were purchased from Dow Corning Corporation (USA). Sulforhodamine B sodium salt and Image-iT® Fixation/Permeabilization Kit were obtained from Sigma-Aldrich, (USA). Fetal bovine serum (FBS) was purchased from Dutscher (France). The poly(methyl methacrylate) PMMA substrates used in this study, thickness 1.1 mm, were purchased from Goodfellow Cambridge Ltd, (UK). The microtools used in the microfabrication process were "tr series 2 flute micro square end mills" with different diameters: 880 and 200 µm purchased from Performance Micro tool (USA). Tubing Silikon Peroxid/60 Shore ID 0.75mm were obtained from IDEX Health & Science Gmbh (USA). Blunt Needle PlasticHub SN-23, 23 G, 0.5" were purchased from Warner Instruments (USA). Micromilling machine was purchased from Minitech Machinery Corporation (USA). Syringe pump PHD ULTRA™ was purchased from Harvard apparatus (USA). MFCS™-EZ: microfluidic flow control system was purchased from Fluigent (France). The human colon adenocarcinoma HT-29 cell line was purchased from ATCC (USA). RPMI 1640 Medium, penicillin-streptomycin solution (10,000 U/mL), trypsin (0.05%), SYTOX® Orange Nucleic Acid Stain, Hoechst, DAPI, Alexa Fluor™ 488 Phalloidin and Calcein AM were purchased from ThermoFisher Scientific Inc (USA).

2.2 Bonding of microfluidic chips

The optimized process for the microfabrication of the chips using this bonding method is schematized in figure 1a. Different patterns were designed and tested: a linear microchannel (880/880 µm, width/depth) and microchannels (200/200 µm) connected to microwells (600/400 µm). First, we micromilled the microstructures onto a PMMA substrates (thickness 1.1 mm), using a CNC micromilling machine (Minitech Machinery Corporation (USA). The PMMA masters were also used to prepare PDMS microchannels by double replica of PDMS. In order to prevent adhesion of the PDMS negative replica on the PDMS master, it was treated with oxygen-plasma, followed by immersion in a silane solution (94% v/v isopropanol, 1% acetic acid, 1% Fluorolink S10, and 4% deionized water) and then placed in an oven at 70 °C for 1 h, thus allowing a complete reaction of the master surface with the fluorinated polymer[34]. The PDMS

was poured into the positive replica and cured at 70°C for 1h to obtain the PDMS microchannels. The PDMS and PMMA microchannels were bonded to different substrates by using the proposed GEL-D method. The NOA sheet (1.5 mm) was prepared pouring the liquid NOA 63 into a square chamber and consequently exposed for 5 min. under UV light. The others bottom parts used were a standard glass slide and a homemade PMMA slide (thickness 1.1 mm). An aqueous solution of gelatin (derived from porcine skin gel strength 300, Type A, Sigma-Aldrich) 15% w/v was prepared, degassed under vacuum and maintained at 70°C. The PDMS, PMMA and NOA substrates (top and bottom parts) were cleaned and dried. The thin gelatin layer onto the substrates (top and bottom) was obtained through sping coating (Laurell, WS-650 Series, 1,500 rpm for 20 sec., acc 500). A typical process involved depositing a small gelatin solution droplet (around 0.5 ml) at the center of the flat and micromilled substrates and then spinning at high speed (1500 rpm for 20 s), as reported in Figure 2~~scheme 1~~. Although it did not necessary for the present study, some types of material (COC or plastic substrates) may require an oxygen-plasma surface treatment prior the spin coating to enhance the uniformity of the deposited layer. After the coating, the gelatin that partially filled the inlet and outlet holes was removed using a biopsy punch. The separate substrates with the hydrated gelatin layer were then clamped (Pony Spring Clamp 210 mm), incubated at 4° C for 10 minutes and dehydrated at room temperature (24°C) for 1-4 days. Assembled chips with different combinations of materials: PMMA/PMMA, Glass/PMMA, Glass/PDMS and NOA/PMMA (figure 1b) were fabricated.

2.3 Leakage test

The sealing of fabricated chips was then evaluated by two different methods: I) by filling the microchannels with a fluorescent liquid to analyze the gray intensity with imageJ (across the microchannel) and II) by osserving the bond burst in a pressure experiment. In particular, a pressure controller MFCS (Fluigent) was used to progressively increase the inlet pressure of 100 mbar every 60 seconds with a constantly closed outlet. The maximum pressures obtained for each configuration before the bond burst are reported in Fig. 5 for either a room temperature environment (ca. 25 °C). All the related results are commented in the “Results and Discussion” section.

2.3 Cell culture on bonded chip

The HT-29 cells were seeded onto the PMMA/PMMA chip bonded with the proposed GEL-D method, as preliminary cell culture application.

HT-29 cells were cultured in RPMI-1640 medium supplemented with 10 % FBS. Cells were sub-cultured, harvested and counted using standard protocols. T-75 flasks of HT-29 and at ~90 % of confluence were used, for all reported experiments. To prevent contamination, the chip was sterilized with 10% penicillin-streptomycin solution in Phosphate-buffered saline (PBS) for 24 h at 4°C. After three washing cycles with PBS buffer, the cell suspensions were injected in the systems using a syringe pump. The cell suspension at a concentration of 5x 10^4 cells/mL was injected at a flow rate of 50 µL/min for 60 sec. After less than 10 minutes under laminar flow hood, the chip was incubated in a 5% humidified CO^2 incubator at 37°C. Cells growth was evaluated, using a confocal microscope. The Pictures were taken after the seeding and at day 1, 2, 3, 4, 5 and 7. The live/dead staining was performed after 1 day of cultivation using Calcein AM and Sytox orange nucleic acid stains at concentrations of 1 µM and 0.250 µM, respectively and cell death visualized with an epifluorescence microscope [35]. The structure analyze after 7 days of culture was performed using a confocal microscope Leica equipped with 4X and 10X objectives. The cells were fixed using a solution of 2% Glutaraldehyde in 0.1M Phosphate Buffer, pH 7.3. After 20 min, the fixative agent was removed and the samples were washed by flowing three times PBS in the microchannel. After permeabilization with 0.5% Triton-X100, the cells were incubated with blocking buffer (1% BSA in PBS) and stained with Phalloidin for 1h at room temperature. DAPI and Hoechst 33342 (1 µL/mL) were used for nuclei staining.

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3 Results and discussion

3.1 Characterization and sealing test

The SEM microscopy was used to analyze the surface structure of the gelatin film, while a profilometer was employed to measure the thickness. For the profilometer characterization, we used a map scan type on valleys profile, a stylus type radius of 2.5 µm and a measurement range of 6.5 µm. As reported in the supplementary material, the gelatin-based coating presents a structure similar to a thin film (Figure S1 a,b) and a depth of 6.2 µm (Figure S1 c). Despite the limited resolution and magnification of the samples due to water content, the SEM micrographs clearly show the absence of micropores that can alter the cell adhesion and culture (Figure S1b). The thin thickness of the gelatin does not considerably alter the initial microchannel configuration (~6 µm) and not clog the microchannel (Fig S1d).

The sealing of these chips was tested by flowing a red fluorescent solution (sulforhodamine B sodium salt 0.1 mg/mL) through a syringe pump (100 µL/min) for 1, 30 and 60 min. As reported in figure 3~~2~~, the liquid remains confined into the microchannels (1 day dehydration chip) and no liquid was observed to spill out of the channel. Nonetheless, some fluorescence is observed outside the channel, indicating that the fluorescence dye has diffused in the gelatin layer (Figure 3~~2~~h). When increasing the dehydration time from 1 day to 3 days before flowing the dye, the fluorescence outside the channel is observed to remain stable to 0, likely indicating that the diffusion of the dye in the interstitial region has been greatly slowed down (Figure 3~~2~~i). This ~~observation~~ result suggests that the permeability of the gelatin layer can be adjusted by choosing the appropriate dehydration time. The influence of the spin coating parameters (spin speed) was also studied, as reported in figure S2. Applying a speed of 1,500 rpm a uniform and regular gelatin thin layer was obtained by guaranteeing the sealing, conversely, when a speed of 2,500 rpm is applied the layer is irregular and this leads the leakage (Fig S2). A parameter that can influence the uniformity of the gelatin layer is the viscosity of the solution. The viscosity of the gelatin dispersion (15% W/V) was than evaluated by using a ……., measuring a value of …… Pa S ~~–~~The strength of the performed bonding was also evaluated by filling the chip with the fluorescent solution and progressively increasing the inlet pressure of 100 mbar every ~~20~~ 60 seconds with a constantly closed outlet (pressure controller MFCS-100, Fluigent). The Pmax obtained before the bond failed are shown in figure ~~4~~2~~j~~. As

observed, the chip prepared by bonding rigid materials such as PMMA/PMMA (0.5 MPa), Glass/PMMA (> 0.7 MPa) and NOA/PMMA (0.6 MPa) fared much better than Glass/PDMS chips (0.08 MPa). Every test has been repeated 3 times. We hypothesized that the great difference between the rigid -and flexible material it depend by……..Nonetheless, all these bond strengths exceed the basic requirements of most of the microfluidic applications. The applicability of the GEL-D bonding method on miniaturized chips (fig. 53a,b) (microchannels width ~200 µm) was also explored. This size range (~800-200 µm) was targeted since it has been described as appropriate for microengineering cells into tissues on biochips.

No leakage was detected even for the narrowest microstructures tested (fig. 53c). The fluorescence intensity outside the microchannel is below the background level of the detector (fig. S3). Confocal microscopy visualizations attest of the good confinement of the red solution inside the microchannel (fig 53d) and microwell (fig 53e). The dye solution remained well-confined into the microchannels. Using z-stack analysis, 3D reconstruction of the microstructures was realized. The results confirm that the GEL-D bonding method can seal chips featuring small microchannels without clogging them, as clearly showed in figure 53h,i.

The chemical resistance of the sealing in the presence of commonly used organic solvents (ethanol and acetone) in microfluidics was also evaluated. These solvents were flowed into the microchannels (880/880 µm) at 100 µL/min for 60 min. After this time, the fluorescence intensity was ~~analysed~~analyzed yielding similar results than when flowing the only fluorescent liquid (fig S4). In addition, the resistance at high temperatures was evaluated (fig S5). After 30 minutes of incubation in a hoven, the red fluorescent liquid was injected in the chip (1 day dehydration). The temperature range tested was 30-80°C, with ramp of 10°C. No leakage was observed for temperature below 70°C. The dehydrated gelatin starts to melt at ~80° C and the chip de-bonds. This technical aspect pave the way for removing the gelatin coating inside the bonded microchannel for other applications, where the gelatin film is not required.

3.2 Cell culture on chip and live/dead assay

Finally, the preliminary applicationbility of the bonded chips for cell culture and characterization after de-bonding was also explored. The durability (ie. the time before which leakage of reagent solution was observed) of the sealing into a cell culture incubator was studied,

considering the rate of gelatin re-hydration compared to the time needed to culture the cells. The chips tested were the PMMA/PMMA and Glass-PDMS (880/880 µm), obtained after 1, 2, 3 and 4 days of dehydration at room temperature. The bonded microchannels were filled with a cell culture medium and incubated at 37°C. The cell culture medium was re-filled on a daily basis, using an injection by micropipette. The results are reported in figure 64a. Every test has been repeated 3 times. The PMMA/PMMA chip obtained after 3 days of dehydration remained sealed until 11 days, while the Glass-PDMS held for 6 days (Figure 64a). ~~This durability is in line with the duration of most microfluidic experiments that is in general 3-7 days. The HT-29 cells were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum. Cells were sub-cultured, harvested and counted using standard protocols. To prevent contamination, the PMMA/PMMA chip was sterilized immersing the device into a 10% penicillin-streptomycin solution in PBS for 15 h at 4°C.~~ After the chip sterilization, ~~T~~the HT-29 cells were seeded onto the PMMA/PMMA chip at a concentration of 10^5 cells/mL. Using a syringe pump the ~~50 µL of~~ cells suspension was injected ~~using a standard pipette~~. The live/dead assay reported in figure 64b shows the vitality of the cells after 1 day of cultivation. The staining was performed on-chip using fluorescent dyes: Calcein AM and Sytox orange nucleic acid stains at concentrations of 1 µM and 0.250 µM, respectively. As showed in figure 46, no dead cells have been detected (Sytox orange), while live cells were stained using Calcein AM. The live/dead assay confirms the biocompatibility of the as-prepared chips.

The applicability to grow on-chip a tumor microtissue was also investigated, extending the cell culture incubation until day 7. The confocal images (fig. 6c) shows a microtissue of around 220 µm in diameter obtained after 7 day of culture. The microtissue fixation was performed on-chip using an Image-iT® Fixation/Permeabilization Kit. Consequently, the fixed microtissue was stained with DAPI (nuclei) and Alexa Fluor™ 488 Phalloidin (actin cytoskeletons). The cell characterizations were carried out after manually de-bonding the chip, as the two parts are easily separable, as reported in the movie 2. We demonstrated that the chip can resist for loading at pressure up to 0.7 mPa. During the cell culture incubation with cell culture medium the chip de-bonded spontaneously after 3, 4, 6 or 11 days depending on the time of dehydration (1, 2, 3 and 4 days) and material combinations. This can be advantageous to simply de-bond the chip when recovery of the cells constructs is needed.

Taken altogether, these findings indicate that: (i) the GEL-D method is useful to bond thermoplastic polymer microfluidics and other materials; (ii) the permeability of the gelatin film can be adjusted operating on dehydration times and (iii) the bonded microchannels are pertinent substrates for cell culture and growth of microtissues, making possible on and off-chip live and post fixation fluorescence imaging.

Conclusions

In this communication, a cost-effective, reproducible and reversible gelatin-based bonding method to fabricate closed microfluidic chips was introduced for the first time. The protocol was validated for a wide range of materials commonly used by the microfluidics community. The unique properties of the biocompatible and cheap gelatin have been exploited to obtain a strong chip sealing. This is, therefore, an interesting alternative to more costly and complex bonding procedures that are often performed in clean room-like facilities, making it suitable and convenient for industrial applications and also for academic practical course. The maximum pressure resistance measured exceeded 0.70 MPa, covering the majority of microfluidic applications. The sealing is also resistant to cell culture conditions and therefore useful in organ and tissue on chip. Furthermore, the possibility to easily de-bond the chip makes it very useful for off-chip cells analysis. The dehydrated gelatin film interface is very transparent and makes possible high quality on-chip imaging and analysis, such as the live/dead assay reported here. Advantageously, the proposed bonding method makes it possible to manufacture

cell culture chips with a gas-permeable intermediate layer, opening new routes for the use of plastic materials instead PDMS. Therefore, we believe that this GEL-D method could be a versatile and simple bonding solution for a wide range of microfluidic platforms, especially pertinent for cell-based applications.

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References

[1] D. J. Beebe, G. A. Mensing, and G. M. Walker, "Physics and applications of microfluidics in biology," *Annual Review of Biomedical Engineering,* vol. 4, pp. 261-286, 2002 2002.

[2] E. W. Young and D. J. Beebe, "Fundamentals of microfluidic cell culture in controlled microenvironments," *Chemical Society Reviews,* vol. 39, no. 3, pp. 1036-1048, 2010.

[3] S. Halldorsson, E. Lucumi, R. Gómez-Sjöberg, and R. M. Fleming, "Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices," *Biosensors and Bioelectronics,* vol. 63, pp. 218-231, 2015.

[4] Y.-X. Huang, C.-L. He, P. Wang, Y.-T. Pan, W.-W. Tuo, and C.-C. Yao, "Versatile on-stage microfluidic system for long term cell culture, micromanipulation and time lapse assays," *Biosensors and Bioelectronics,* vol. 101, pp. 66-74, 2018.

[5] K. Ziolkowska, E. Jedrych, R. Kwapiszewski, J. Lopacinska, M. Skolimowski, and M. Chudy, "PDMS/glass microfluidic cell culture system for cytotoxicity tests and cells passage," *Sensors and Actuators B: Chemical,* vol. 145, no. 1, pp. 533-542, 2010.

[6] H. Becker and C. Gartner, "Polymer microfabrication technologies for microfluidic systems," (in English), *Analytical and Bioanalytical Chemistry,* Review vol. 390, no. 1, pp. 89-111, Jan 2008.

[7] O. Rotting, W. Ropke, H. Becker, and C. Gartner, "Polymer microfabrication technologies," (in English), *Microsystem Technologies,* Article; Proceedings Paper vol. 8, no. 1, pp. 32-36, Mar 2002.

[8] H. Becker and C. Gartner, "Polymer microfabrication methods for microfluidic analytical applications," *Electrophoresis,* vol. 21, no. 1, pp. 12-26, Jan 2000.

[9] Y. Temiz, R. D. Lovchik, G. V. Kaigala, and E. Delamarche, "Lab-on-a-chip devices: How to close and plug the lab?," *Microelectronic Engineering,* vol. 132, pp. 156-175, 2015.

[10] S. Bhattacharya, A. Datta, J. M. Berg, and S. Gangopadhyay, "Studies on surface wettability of poly(dimethyl) siloxane (PDMS) and glass under oxygen-plasma treatment and correlation with bond strength," *Journal of Microelectromechanical Systems,* vol. 14, no. 3, pp. 590-597, Jun 2005.

[11] N. Y. Lee and B. H. Chung, "Novel poly (dimethylsiloxane) bonding strategy via room temperature “chemical gluing”," *Langmuir,* vol. 25, no. 6, pp. 3861-3866, 2009.

[12] C.-W. Tsao and D. L. DeVoe, "Bonding of thermoplastic polymer microfluidics," *Microfluidics and Nanofluidics,* vol. 6, no. 1, pp. 1-16, 2009.

[13] D. J. Harrison, A. Manz, Z. Fan, H. Luedi, and H. M. Widmer, "Capillary electrophoresis and sample injection systems integrated on a planar glass chip," *Analytical chemistry,* vol. 64, no. 17, pp. 1926-1932, 1992.

[14] Z.-J. Jia, Q. Fang, and Z.-L. Fang, "Bonding of glass microfluidic chips at room temperatures," *Analytical chemistry,* vol. 76, no. 18, pp. 5597-5602, 2004.

[15] S. Silvestrini *et al.*, "Tailoring the wetting properties of thiolene microfluidic materials," *Lab on a Chip,* vol. 12, no. 20, pp. 4041-4043, 2012.

[16] L. Tang and N. Y. Lee, "A facile route for irreversible bonding of plastic-PDMS hybrid microdevices at room temperature," *Lab on a Chip,* vol. 10, no. 10, pp. 1274-1280, 2010.

[17] R. Gajasinghe *et al.*, "Experimental study of PDMS bonding to various substrates for monolithic microfluidic applications," *Journal of Micromechanics and Microengineering,* vol. 24, no. 7, p. 075010, 2014.

[18] V. Sunkara, D.-K. Park, H. Hwang, R. Chantiwas, S. A. Soper, and Y.-K. Cho, "Simple room temperature bonding of thermoplastics and poly (dimethylsiloxane)," *Lab on a Chip,* vol. 11, no. 5, pp. 962-965, 2011.

[19] J. Wu and N. Y. Lee, "One-step surface modification for irreversible bonding of various plastics with a poly (dimethylsiloxane) elastomer at room temperature," *Lab on a Chip,* vol. 14, no. 9, pp. 1564-1571, 2014.
[20] P.-C. Chen and L. H. Duong, "Novel solvent bonding method for thermoplastic microfluidic chips," *Sensors and Actuators B: Chemical,* vol. 237, pp. 556-562, 2016.
[21] S. A. M. Shaegh *et al.*, "Rapid prototyping of whole-thermoplastic microfluidics with built-in microvalves using laser ablation and thermal fusion bonding," *Sensors and Actuators B: Chemical,* vol. 255, pp. 100-109, 2018.
[22] K. Anwar, T. Han, and S. M. Kim, "Reversible sealing techniques for microdevice applications," *Sensors and Actuators B: Chemical,* vol. 153, no. 2, pp. 301-311, 2011.
[23] M. Serra, I. Pereiro, A. Yamada, J.-L. Viovy, S. Descroix, and D. Ferraro, "A simple and low-cost chip bonding solution for high pressure, high temperature and biological applications," *Lab on a Chip,* vol. 17, no. 4, pp. 629-634, 2017.
[24] T. Yamashita, Y. Tanaka, N. Idota, K. Sato, K. Mawatari, and T. Kitamori, "Cultivation and recovery of vascular endothelial cells in microchannels of a separable micro-chemical chip," *Biomaterials,* vol. 32, no. 10, pp. 2459-2465, 2011.
[25] G. Pitingolo, P. Nizard, A. Riaud, and V. Taly, "Beyond the on/off Chip Trade-off: a Reversibly Sealed Microfluidic Platform for 3D Tumor Microtissue Analysis," ed. Sensors & Actuators: B. Chemical, 2018.
[26] D. Bartolo, G. Degré, P. Nghe, and V. Studer, "Microfluidic stickers," *Lab on a Chip,* vol. 8, no. 2, pp. 274-279, 2008.
[27] E. P. Dupont, R. Luisier, and M. A. M. Gijs, "NOA 63 as a UV-curable material for fabrication of microfluidic channels with native hydrophilicity," (in English), *Microelectronic Engineering,* Article; Proceedings Paper vol. 87, no. 5-8, pp. 1253-1255, May-Aug 2010.
[28] H. Wu, B. Huang, and R. N. Zare, "Construction of microfluidic chips using polydimethylsiloxane for adhesive bonding," *Lab on a Chip,* vol. 5, no. 12, pp. 1393-1398, 2005.
[29] S. Satyanarayana, R. N. Karnik, and A. Majumdar, "Stamp-and-stick room-temperature bonding technique for microdevices," *Journal of Microelectromechanical Systems,* vol. 14, no. 2, pp. 392-399, 2005.
[30] S. R. Caliari and J. A. Burdick, "A practical guide to hydrogels for cell culture," *Nature methods,* vol. 13, no. 5, p. 405, 2016.
[31] V. van Duinen, S. J. Trietsch, J. Joore, P. Vulto, and T. Hankemeier, "Microfluidic 3D cell culture: from tools to tissue models," *Current opinion in biotechnology,* vol. 35, pp. 118-126, 2015.
[32] G.Pitingolo, V.Taly, and C. Nastruzzi. (2018). *A second life for old electronic parts: a spin coater for microfluidic applications*.
[33] R. Vecchione, G. Pitingolo, A. P. Falanga, D. Guarnieri, and P. A. Netti, "Confined gelatin dehydration as a viable route to go beyond micromilling resolution and miniaturize biological assays," *ACS applied materials & interfaces,* 2016.
[34] L. G. Occhipinti, "Surface treatment of an organic or inorganic substrate for enhancing stability of a lithographically defined deposited metal layer " vol. US Pat. App. 12/835,011 F. Porro, Ed., ed: US Pat. App. 12/835,011 2010.
[35] E. Brouzes *et al.*, "Droplet microfluidic technology for single-cell high-throughput screening," *Proceedings of the National Academy of Sciences,* vol. 106, no. 34, pp. 14195-14200, 2009.

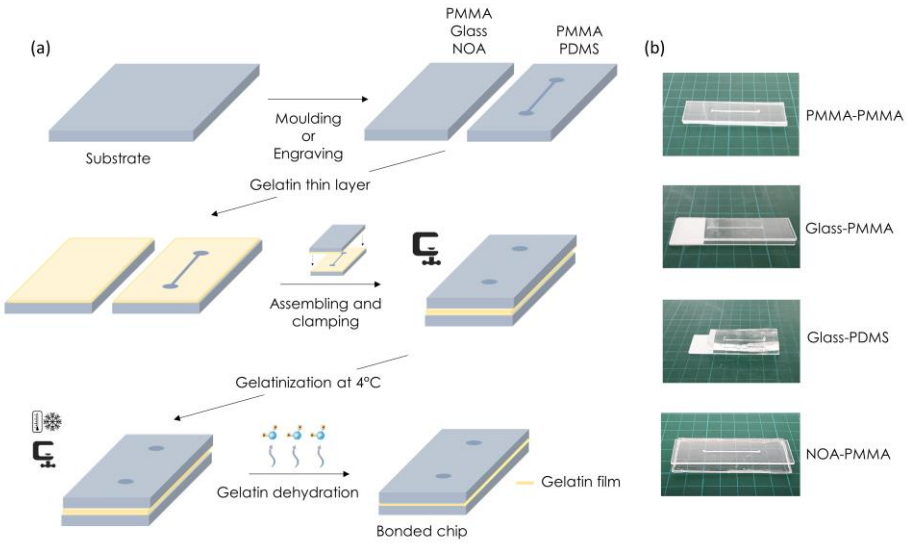

Figure 1

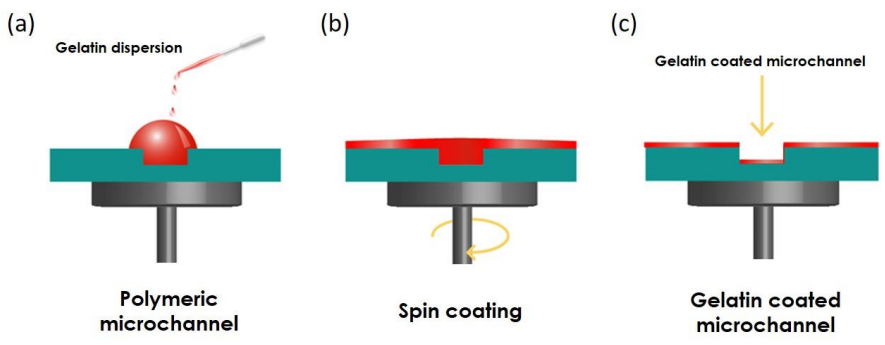

Figure 2

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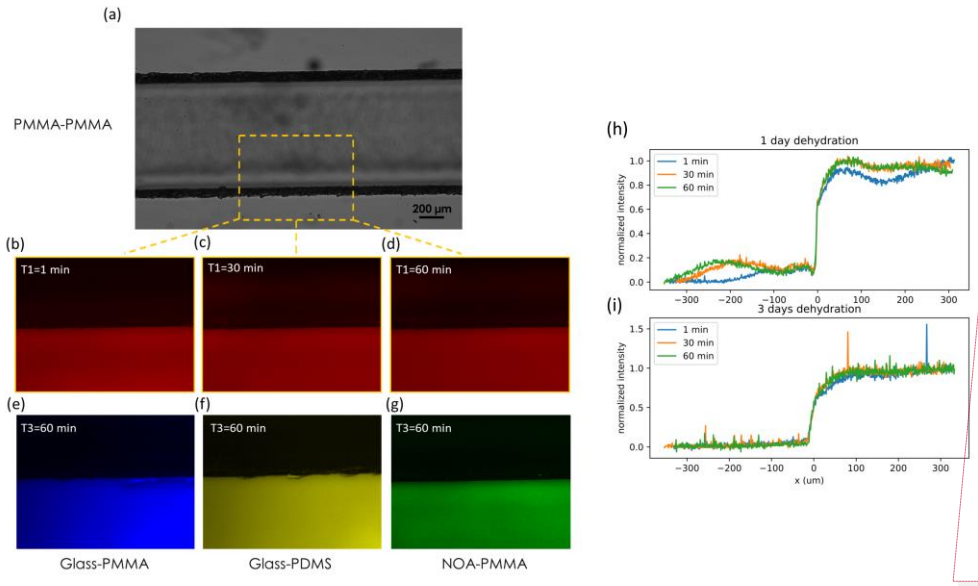

Figure 3

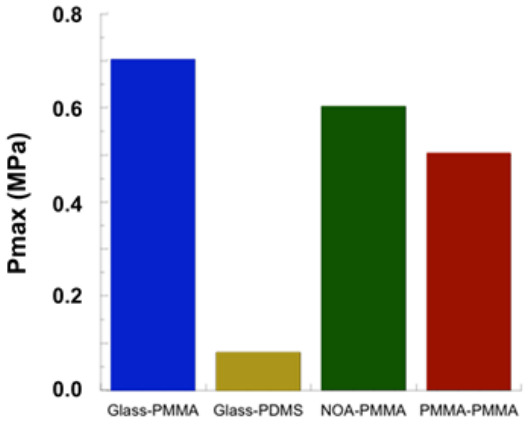

Figure 4

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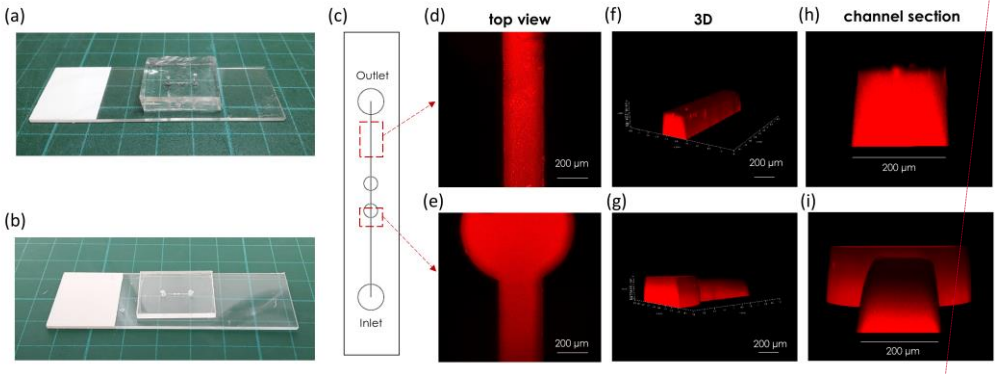

Figure 5

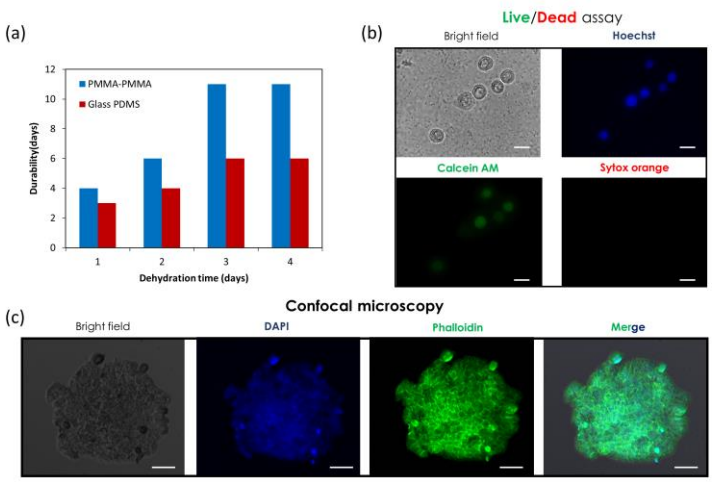

Figure 6

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Figure legends

Figure 1. Microfabrication protocol and bonded chips. (a) Schematic illustration of the GEL-D bonding method. (b) The pictures of the chips fabricated using the proposed method.

Figure 2~~Scheme 1~~. Scheme of the gelatin coating process. (a) Deposition of gelatin dispersion, (b) thin layer formation by spin coating technology and (c) gelatin coated microchannel ready for the "assembling/clumping" step.

Figure ~~2~~3. Sealing test of bonded chips. The red fluorescent solution was injected at 100 µL/min. (a) A bright field microscopy image of the PMMA/PMMA microchannel filled with the liquid solution. (b-d) Epi-fluorescence images of the half-part PMMA/PMMA microchannel after 1 (b), 30 (c) and 60 (d) minutes of flow injection. (e-g) Epi-fluorescence images after 60 minutes of flow into the other configurations: Glass/PMMA (e), Glass/PDMS (f) and NOA/PMMA (g). (h-i) Analysis of the fluorescent intensity across the microchannels (after 60 min of flow) in the chip bonded after 1 (h) and 3 (i) days of dehydration.

Figure 4~~(j)~~ Bond strength of the proposed method for different configuration chips. The pressures at which a bonding burst occurred are shown for each chip. The highest tested pressure was 0.7 MPa.

Figure 5~~3~~. Bonding of narrower microchannels. (a-b) Photograph of the bonded chip featured with linear microchannels connected to microwells. (c) The detailed design developed to show the versatility of the GEL-D bonding method. (d-e) Top view of the fluorescent solution confined in the miniaturized channel. (f-g) A 3D reconstruction of confocal microscopy images showing that gelatin not alter the initial configuration. (h-i) Relative sections of the microchannel connected to the microwell.

Figure 6~~4~~. Application of the fabricated chips for cell culture. (a) The graph shows different durability times in a cell culture incubator for the PMMA/PMMA and Glass/PDMS chips bonded with a dehydration time of 1, 2, 3 and 4 days. (b) Live/dead assay of HT-29 cells after 1 days of culture, scale bar 10 µm. (c) Growth of a tumor microtissue after 7 days of culture on-chip, scale bar 50 µm. The confocal microscopy characterization was made off-chip, after the de-bonding of the device.