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Advanced Micromachining of Concave Microwells for Long Term On-Chip Culture of Multicellular Tumor Spheroids

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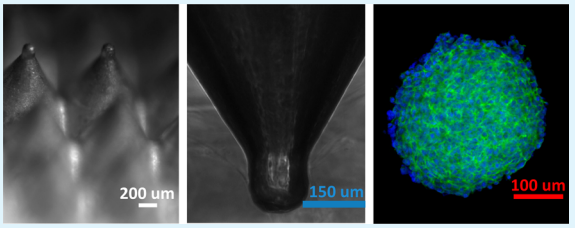
Supporting Information

ABSTRACT: A novel approach based on advanced micromachining is demonstrated to fabricate concave microwell arrays for the formation of high quality multicellular tumor spheroids. Microfabricated molds were prepared using a state-of-the-art CNC machining center, containing arrays of 3D convex micropillars with size ranging from 150 μm to 600 μm. Microscopic imaging of the micropillars machined on the mold showed smooth, curved microfeatures of a dramatic 3D shape. Agarose microwells could be easily replicated from the metallic molds. EMT-6 tumor cells seeded in the primary macrowell sedimented efficiently to the bottom of the concave microwells and formed multicellular spheroids within 48 h. Dense and homogeneous multicellular spheroids were obtained after 10 days of culture, confirming the suitability of the proposed approach. To facilitate long term spheroid culture and reliable on-chip drug assay, polydimethylsiloxane microwells were also replicated from the metallic molds. A solvent swelling method was adapted and optimized to Pluronic F127 towards physically entrapping the block copolymer molecules within the polydimethylsiloxane network and in turn to improve long term cell-binding resistance. Homogeneous multicellular spheroids were efficiently formed in the concave microwells and on-chip drug assays could be reliably carried out using curcumin as a model anti-cancer drug. Advanced micromachining provides an excellent technological solution to the fabrication of high quality concave microwells.



KEYWORDS: *multicellular tumor spheroids, micromilling, microwells, polydimethylsiloxane, pluronics, drug screening*

INTRODUCTION

Three dimensional (3D) tumor tissues are an *in vitro* improved model over standard 2D culture that better mimics the physiological and biochemical environment of tumor *in vivo*. In particular, it provides cancer cells with the relevant cell−cell and cell−extracellular matrix (ECM) interactions.[1] Tumor spheroids assemble as functional units of tumor tissues with cell−cell interactions, cell−extracellular matrix (ECM) signalling, and tumor-like radial physiological gradients.[2] The biological similarity of the 3D tumor model with pathophysiological profiles, in term of gene and protein expression, proliferation, and gas/nutrient gradients, makes tumor spheroids an excellent platform to understand biophysiological microenvironment, as well as to screen pharmaceutical candidates.[3,4]

However, commonly used methods based on the hanging drop and spinner flask approaches are limited in their abilities to prepare large numbers of spheroids with good uniformity, which are required for standard biochemical assays.[5] In addition these methods are typically not compatible with imaging and analytical high throughput on-chip assays. Considerable research efforts have, therefore, been made in recent time to develop improved tumor spheroid preparation technologies towards their application in cancer pre-clinical research. For instance, high throughput hanging drop plates, designed to overcome these drawbacks,[6−8] are now commercially available in a 384 format enabling high throughput formation and manipulation of multicellular spheroids. Conversely, the application of microfabrication technology has offered new solutions for the fabrication of microwells compatible with high throughput preparation of multicellular tumor spheroids with well-defined sizes and shapes.[9] Recent reports demonstrate that the ideal characteristics of microfabricated microwells for tumor spheroid formation include concave shapes and cell binding resistant surfaces, which promote the formation of cellular aggregates. However, the preparation of concave microwells, especially at the scale required for high throughput spheroid-based biological assays remains a technical challenge. Lithography and laser ablation, the most common direct substrate manufacturing technologies, are typically not compatible with the formation of curved surfaces at the microscale. Two approaches have been recently advanced to fabricate high quality concave microwells for spheroid culture, namely the surface tension-mediated fabrication of PDMS microwells[10] and the pressure enhanced deflected method initially reported by Choi et al.[11−14] A simpler and more flexible alternative is the use of microfabricated molds that can be replicated using materials compatible to the formation of spheroids. To this end, rapid prototyping 3D printing technologies have been successfully applied to fabricate wax

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molds, which can in turn be used to prepare siloxane-based elastomer molds that could ultimately be replicated using agarose to prepare microwells.[15] Despite the advancement of 3D printing technologies, they remain limited for the preparation of small structures in regards to the surface finish. Agarose microwells replicated from microfabricated elastomer molds provide nevertheless a very robust technology for the preparation of multicellular spheroids and have become commercially available.

Computer numerical control (CNC)-based mechanical micromachining or micromilling is a powerful rapid prototyping technology with sub-micrometer-level accuracy able to fabricate micrometer-scale structures with high curvatures and excellent surface finishes. Micromilling is compatible with a broad range of substrates, including metals, and micromachined molds are typically resistant to wear, mechanically robust, compatible with harsh solvents, and accordingly provide prolonged periods of reusability. In addition, metallic micromachined molds can withstand high temperature and high pressure, depending on the materials used, and thus can be combined with either soft lithography, hot embossing, or injection molding processes.[16,17]

Owing to its inherent high cell-binding resistance, ease of use and suitable mechanical integrity, agarose is a material of choice in replication-based approaches to prepare microwells. Agarose is commonly used as non-adherent coating for 96-well plates, which provides a very simple method for the preparation of multicellular spheroid. However, agarose and, more generally, hydrogel-based materials are not ideal for spheroid-based drug assays because small (bio)molecules can diffuse inside the gels and cause inaccuracy in drug treatment. In addition, long term culture in agarose microwells is challenging as the material tends to lose its mechanical integrity over the time. Polydimethylsiloxane (PDMS) elastomer replicas are also commonly used for the fabrication of microwells from molds produced by conventional processes. PDMS is cross-linked and chemically inert and is the most widely used replication materials in microfluidics. However, PDMS has to be surface modified to avoid the physical adsorption of proteins and biomolecules as well as cellular binding. Among the many established PDMS passivation strategies,[18] coatings with PEO-tethered layers, including the physical adsorption of Pluronic block copolymers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO−PPO−PEO) is a good compromise between performance and simplicity.[19,20] The hydrophobic PPO block is prone to interact the PDMS hydrophobic surface, while the hydrophilic PEO blocks tend to stretch into the aqueous phase and form biomimetic "nanocilia", which can repel protein and cell attachment.[20−23] However, physically adsorbed block copolymers can be easily displaced from the surface, limiting the long term performance of block copolymer based cell-resistant PDMS.[24,25] A simple yet efficient method to enhance the stability of PEO-tethered layers is to physically entrap them within the PDMS network. This can be achieved using solvent induced swelling the of PDMS as demonstrated using a poly(dimethylsiloxane)-*b*-poly(ethylene oxide) diblock copolymer.[26]

The present study aims to establish a robust microfabricated approach towards the formation of high quality multicellular tumor spheroids based on micromachined molds. We machine metallic molds using a state-of-the-art 3-axis CNC machining center to achieve arrays of 3D convex structures with dimensions ranging from 150 μm to 600 μm which could be replicated to yield concave microwells with excellent surface finishes. Using these molds, we cast agarose microwells and use them to grow high quality multicellular spheroids using the mammary carcinoma cell line EMT-6. Towards enabling long term spheroid culture and on-chip reliable drug assay, PDMS microwells were prepared using the micromachined molds. To improve the cell-binding resistance of the PDMS microwells, a solvent-induced physical entrapment method was developed to incorporate Pluronic F127 block copolymer molecules within the polydimethylsiloxane network. The method relies on swelling the PDMS elastomer in an appropriate solvent in the presence of the amphiphilic F127 block copolymer, which is then trapped within the polymer upon rapid de-swelling resulting from a change in solvent. Homogeneous multicellular spheroids are efficiently formed in the concave microwells and their suitability for on-chip drug assays is determined using curcumin as a model anti-cancer drug.

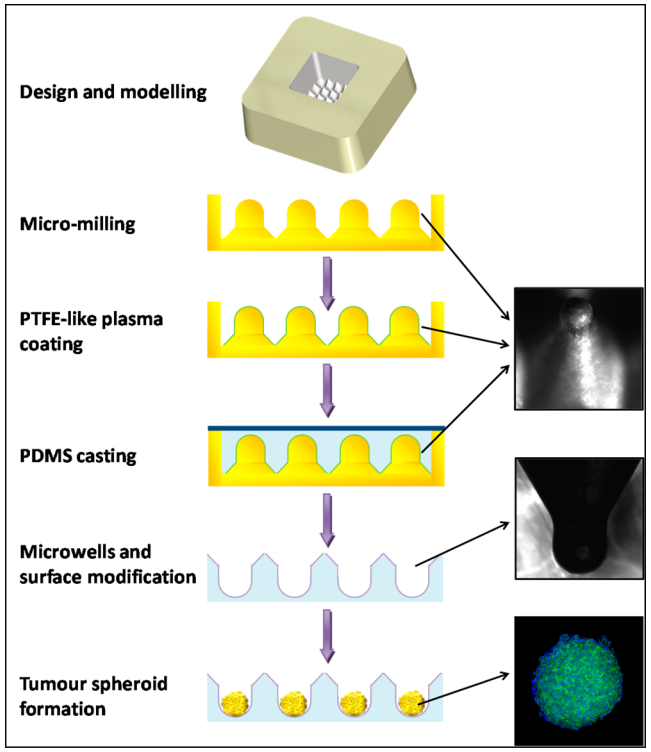
EXPERIMENTAL PROCEDURE

Micromachining of Metallic Molds. A mold was designed to accommodate the simultaneous casting of three different diameters of microwells (150 μm, 350 μm, and 600 μm) in rectilinear arrays at the base of much larger macrowells capable of receiving up to 0.5 mL of cell suspension. As shown in Scheme 1, each individual well was shaped so that the aspect ratio (well depth/well diameter) of the confined part of the well for spheroid growth was constant at a value of one, regardless of the well diameter. This was done so that the diffusion of solutes in the proximity of the spheroid was constant for each of the well diameters relative to the bulk medium. For all microwells, the base of the well was hemispherical in shape with parallel side-walls. A steep, conical sidewall draft of 60 degrees (included) angle leading into each microwell allowed cells from bulk suspension to sediment into the microwell structure without becoming stuck. In effect, each array of microwells was an array of adjoining circular funnels, the base of which formed a hemispherically shaped microwell of aspect ratio, 1. The overall well structure ensured that all cells sedimenting from the bulk suspension contained by the macrowell found their way to the base of a microwell in equal distribution. The mold superstructure incorporated a microscope slide spacer, offset from the hemispherical pillar tips by 200 μm so that the cast elastomer could be finished with a smooth, flat base only 200 μm thick at the base of each microwell. This was to allow for easy visualisation of tumor spheroids.

After modelling (Solidworks, Dassault Systèmes) and subsequent machine programming (SolidCAM) the mold was machined from aluminum alloy 6061 using a Supermill-2M, 3-axis machining center (Kira Corporation, Japan) with a Fanuc 31i-Series A controller allowing 10 nm positional accuracy on each axis with simultaneous interpolation. After roughing operations and finishing of the macroscaled features, the microstructure was finished with a 152 μm diameter ball-nosed, cemented micrograin tungsten carbide cutter (Performance Micro Tools, U.S.A.). The finish was climb-cut at 8 μm depth with a 1.4% tooth load at 1.4×10^5 rpm (cutting speed 67 m/min) and cooling by oil-mist (Plantokut Mikro 10SR, Fuchs). Constant z-level finishing was performed so that the maximum cusp height between cutter passes on the microstructure was 200 nm. The hemispherical ends of the pillars were very gently polished with 4 μm aluminum dioxide suspension-impregnated cotton wool to remove any nano-sized machining burrs.

To facilitate the release of the replica from the mold, a polytetrafluoroethylene (PTFE)-like thin and conformal anti-sticking layer was deposited through plasma polymerization. The monomer (trifluoromethyl)undecafluorocyclohexane was plasma polymerized in a custom-built reactor setup reported previously.[27] The surface chemistry of the PTFE-like coating was characterized using a Kratos AXIS Ultra DLD X-ray photoelectron spectrometer (XPS) equipped with a monochromatic Al Kα X-ray source ($h\nu$ = 1486.69 eV) and a hemispherical analyser. The binding energies were referenced to the C

Scheme 1. Fabrication Process of the Concave Microwells Array[a]



[a]Metallic molds are first fabricated using advanced CNC micromachining. Design contains a macrowell at the bottom of which is the microwell array (in a typical design: 7 × 7 150 μm microwells, 6 × 6 350 μm microwells, or 5 × 5 600 μm microwells). The metallic molds are replicated with either agarose or PDMS and surface modify with the F127 Pluronic when required (PDMS microwells) to reduce cell-binding. Cells seeded in the macrowell sediments homogeneously within the concave microwells and formed multicellular spheroids within typically 48 h of culture. Long term culture, on-chip drug assays as well as real-time high resolution confocal microscopy imaging are possible in the PDMS microwells.

1s hydrocarbon carbon peak at 285.0 eV to compensate for surface charging effects. Component fitting of high resolution spectra was performed using CasaXPS version 2.3.12 software. Shirley-type backgrounds were used and components constrained to a full width at half-maximum between 0.9 and 1.5 eV. The peak fits used 70% Gaussian/30% Lorentzian peak shapes.

Preparation of Agarose Microwells. Agarose solution (2 wt %) was prepared by autoclaving in Dulbecco's Modified Eagle's Medium (DMEM). The sterilize agarose solution was then poured onto the micromachined metallic molds. A glass slide was placed on the spacer of the molds to provide a flat thin bottom. After setting of the agarose solution, the agarose microwells were gently released from the molds and kept in cell culture medium prior to use.

Pluronic F127 Surface Modification of PDMS for Long Term Cell Repellence. The swelling method initially described for the poly(dimethylsiloxane)-*b*-poly(ethylene oxide) diblock copolymer was optimized for Pluronic F127 towards achieving long term cell repellence and compared with standard physical adsorption. Thin layers of PDMS (SYLGARD 184, Dow Corning, U.S.A.) were first spin-coated on glass coverslips with a ~100 μm thicknesses and baked for 1 h at 65−70 °C. For physical adsorption, the Pluronic F127 solution (1% w/v in water) was incubated on the PDMS coated coverslips for 12 h. To establish the efficiency of the swelling approach to Pluronic F127, the spin-coated glass coverslips were first immersed in F127 solution in acetone (1:1 v/v) for 12 h. The samples were then quickly washed in water and dried in a vacuum oven at 80 °C to induce deswelling of the PDMS network. Alternatively, sequential adsorption and swelling was carried on as follow. F127 was first adsorbed on the PDMS from an aqueous solution after which the samples were quickly immersed in acetone for 2 h. The samples were then rapidly rinsed with water and placed in a dried at 80 °C for 12 h under vacuum conditions.

All samples were thoroughly rinsed with MilliQ water and dried with nitrogen prior to use. Untreated PDMS was used as a control. To ascertain the extent of absorption of the F127 Pluronic, water contact angles were determined using the sessile drop method. The surface chemistry of the treated PDMS samples was determined by XPS analysis as described above.

To test the long term cell-binding resistance, a cell adhesion assay was carried on for 3 days. The mammary carcinoma cell line EMT-6 was cultured in complete media DMEM consisting of supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in a humidified incubator at 37 °C, 5% CO_2, and 100% humidity. EMT-6 cells (1×10^5 cells/mL, 500 μL) were seeded on the samples using custom made incubation chambers and placed in a cell culture incubator for 3 days. Samples were then washed with PBS, fixed using cold methanol for 10 min and stained with DAPI. Cells attached on the surfaces were counted under an inverted microscope in 10 fields of view randomly chosen. The cell resistance percentage was calculated based on the untreated PDMS surface.

Preparation of PDMS Microwells. PDMS was prepared by mixing the elastomer and the curing agent with ratio of 10:1 (w/w). The mixture was poured into the micromachined metallic mold and a glass slide was placed on the spacer of the molds to provide a flat and thin bottom. After degassing under vacuum and baking at 65 °C for 2 h, the PDMS replica was peeled from the mold and cleaned with ethanol several times. The surface of the PDMS was subsequently passivated using F127 using the optimized swelling method.

Tumor Spheroid Formation in the Concave Microwells. Both agarose and Pluronic F127 modified PDMS microwells were first equilibrated with cell culture medium for 30 min before cell seeding. Single cell suspensions of EMT-6 cells (1×10^5 cells/mL, 200 μL) were seeded into the devices primary macrowell and left to sediment with gentle orbital agitation. Culture medium was removed every 2 days and replenished with fresh medium. Microwell arrays were kept in an incubator at 37 °C, 5% CO_2, and 100% humidity for 10 days and aggregation and growth of the cells was monitored regularly using inverted light microscope (Nikon Eclipse TE, Japan).

At the desired time points, the spheroids were fixed on-chip with 4% formaldehyde for 1 h and then permeabilized with 0.1% Triton X-100 in PBS for 1 h. The F-actin and nuclei of the cells were then stained with Rhodamine Phalloidin (50 μg/mL) and DAPI (1 μg/mL) in the dark at room temperature for 1 h. Spheroids inside the microwells were then washed with PBS to remove excess fluorescent dyes before being imaged with an inverted Nikon A1R laser scanning confocal microscope. The spheroids were scanned in the *z*-direction with a step size of 2 μm.

On-Chip Drug Assay Using Curcumin. EMT-6 multicellular spheroids were cultured in the Pluronic F127 treated microwells for 10 days prior to drug treatment. Curcumin stock solution (100 mM) was prepared in DMSO and stored at −80 °C prior to use. Tumor spheroids were incubated with curcumin (100 μM) or DMEM media with 0.1% (v/v) DMSO as control for 24 h. Cell viability was determined by counting live and dead cells using the LIVE/DEAD assay. Live cells were stained with Calcein (2 μM in cell culture media), while dead cells were stained with Ethidium Homodimer (4 μM in cell culture media). Fluorescent images of stained spheroids inside of the microwells were taken with a Nikon A1R laser scanning confocal microscope using a 20× air objective. Images were acquired approximately 100 um inside the spheroids to obtain representative data. Live/dead cell counting was performed using the cell counting function of Nikon confocal microscope software.

RESULTS AND DISCUSSION

Micromilling and Fabrication of the Microwell Arrays. The design of the microwell arrays contains two key elements: (1) a primary macrowell that can be loaded with cell suspensions and culture medium and (2) an array of concave microwells with funnel-shaped opening and diameters of 600 μm, 350 μm, or 150 μm at the bottom of the macrowell. The "funnel shape" of the microwells opening was designed not only to efficiently collect cells seeded in the primary macrowell but also to minimize the loss of spheroids during media manipulation. Complex 3D metallic molds of micropillars with different size were machined in order to reproduce the negative structure as microwell arrays (Figure 1). The microscopic image of the micropillars machined on the mold showed smooth, curved microfeatures of a dramatic 3D shape. For this application, high resolution micromachining of metallic molds has several advantages over other microstructure fabrication technologies. Firstly, convex/concave shapes with dimensions in the range of hundreds of micrometres can be easily machined. The flexible nature of CNC machining means that the particular geometry of the microstructure is only limited by mold ejection. This benefit is made obvious by the vast number of consumer products fabricated by the plastic injection molding process. The use of accurately micromachined true 3D structures in metals with high temperature resistance and mechanical yield strength also reflects common high throughput fabrication technologies such as hot embossing and injection molding. This makes the commercial translation of laboratory outputs more readily achieved. On the other hand, standard microfabrication methods such as photolithography and laser ablation are mostly limited to the fabrication of 2D structures. In addition, despite tremendous advances, rapid prototyping with 3D printing technologies remains limited by its lack of printing resolution and poor surface finish at the dimensions required for spheroid forming microwells. Rapid prototyping could nevertheless be successfully used to fabricate elastomer molds suitable for large scale and reliable replication of agarose microwells.[15]

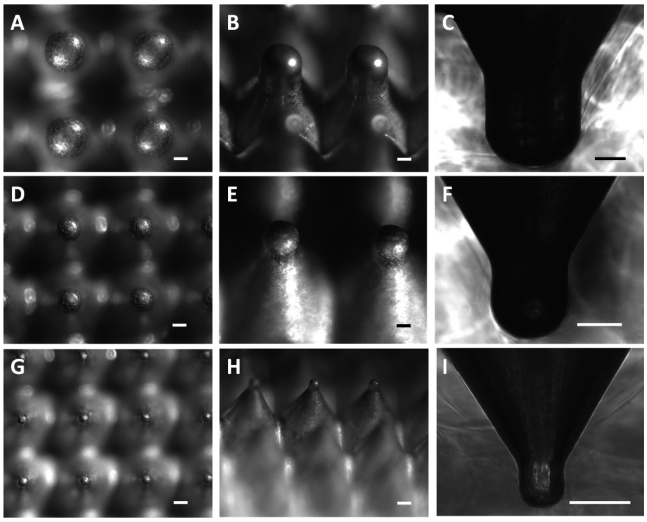


Figure 1. Microscopic imaging of 600 μm metallic micropillars: (A) top view and (B) side view of the pillars. (C) Cross-section image of a 600 μm microwell replicated from the metallic mold. Microscopic imaging of 350 μm metallic micropillars: (D) top view and (E) side view of the pillars. (F) Cross-section image of a 350 μm microwell replicated from the metallic mold. Microscopic imaging of 150 μm metallic micropillars: (G) top view and (H) side view of the pillars. (I) Cross-section image of a 150 μm microwell replicated from the metallic mold. Scale bars are 200 μm.

When compared to the recently described concave microwell microfabrication approaches, the use of micromachined metallic mold and soft replication is also advantageous. SU-8 masters prepared using pressure enhanced deflected structures have been developed to form concave microwells.[11−13] However, microwells' design is inherently limited by the applicable negative pressure and the elasticity of PDMS.[11] Importantly, only shallow microwells can be prepared using this method, and long term on-chip culture as well as on-chip assay is therefore very challenging. In addition, metallic molds are wear-resistant and can be accurately reproduced, whereas SU-8 masters have only limited lifetime. Another fabrication method was recently proposed based on the meniscus-induced self-organization of concave microwells that were successfully used for large scale preparation of homogeneously sized embryoid bodies.[10] Despite the merits of this approach, only narrow microwells can be prepared, thereby limiting its applicability for long term spheroid culture and on-chip assays.

Despite the inherent advantages of metallic molds, it is necessary to apply a non-sticking coating to facilitate the release of the replicated microwell array. While agarose structures could be peeled off from the molds, a PTFE-like non-sticking coating was necessary to release PDMS replicas. This was achieved using plasma polymerization of the (trifluoromethyl)-undecafluorocyclohexane monomer, which enables the uniform deposition of a thin and conformal fluorinated polymeric layers within the complex structure of the mold. The successful deposition of the fluorinated plasma polymer surface chemistry was confirmed using XPS, which showed the typical chemistry as revealed in the elemental and C 1s high resolution analyses (Supporting Information Figure IS1). PTFE-like coatings deposited by plasma polymerization have been previously successfully used as anti-adhesive layer to facilitate the demolding process in microfabrication.[28,29]

Optimization of Pluronic F127 Coatings. While direct physical adsorption of PEO-tethered polymers such as Pluronics is a well-established method to increase its cell-binding resistance, the effect is only transient and the block copolymers are easily displaced over time. A swelling/deswelling method has been developed to physically trapped a poly(dimethylsiloxane)-*b*-poly(ethylene oxide) diblock copolymer within the polydimethylsiloxane network.[26] In this method, copolymer chains become physically trapped within the PDMS elastomeric network upon rapid deswelling resulting from a change in solvent. Building on this concept, the swelling/deswelling method was applied to the F127 Pluronic towards achieving the long term cell-binding resistance required for long term on-chip culture of the spheroid in PDMS microwells. The hydrophilicity of the treated PDMS was examined using static water contact angle measurement. Contact angles were significantly decreased on PDMS samples treated with the F127 polymer using the swelling/deswelling method (57 ± 4 degree) compared with untreated PDMS (119 ± 1 degree) (Figure 2A). Direct adsorption in water (non-swelling condition) of Pluronic F127 also reduced the water contact angle, although to a much lower extent (113 ± 1 degree). A hybrid method combining direct physical adsorption of F127 then swelling of PDMS was also efficient to increase the hydrophilicity of the treated PDMS (82 ± 3 degree),

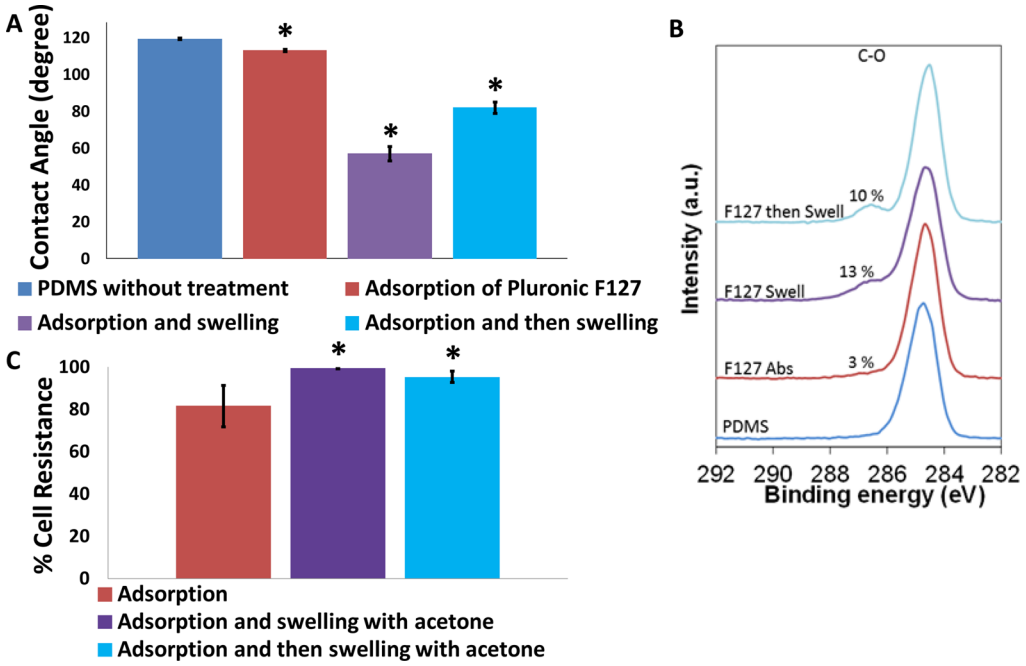


Figure 2. (A) Static water contact angles of F127 Pluronic modified PDMS surfaces. Data shown as mean contact angle (degree) ± standard deviation. (B) High resolution XPS C 1s spectra of the Pluronic modified PDMS surfaces showing the percentage of C−O contribution associated with the presence of the PEO side chains. (C) Cell-binding resistance percentage (native PDMS is used as the reference). Asterisks (*) indicate statistical significance compared to control at $p < 0.05$ using Student's t tests.

although not the same extent than the one-step swelling method. These results confirmed that the swelling method could be efficiently applied to form a dense layer of F127 on PDMS and that the tethered PEO chains adopted a suitable conformation, extending in the liquid phase.

The amount of Pluronic F127 grafted on the PDMS surface after treatment was also quantified using XPS analysis by comparing the area under the C−O peak associated with the PEO ether units versus the $(CH_3)_2SiO$ groups characteristic of poly(dimethylsiloxane) in the C 1s high resolution spectra (Figure 2B). The percentages of C−O group on the surface were 3%, 13%, and 10% for Pluronic F127 adsorption, Pluronic F127 adsorption and swelling, and adsorption and then swelling, respectively. The XPS data was therefore consistent with contact angle measurements and confirmed that the swelling approach can be used to achieve high coverage of F127 Pluronics on PDMS. Similar trapping of Pluronic F108 was successfully achieved using the swelling−deswelling approach to graft PEG with controllable chains density onto 2D and 3D SU-8 patterns.[30] As an alternative to the swelling method, the method described by Wu et al., which relies on the migration of PEO chain in the liquid phase from a bulk hybrid material prepared using a mixture of PDMS and Pluronic F127, was also tested.[31] The incorporation of Pluronics within the bulk PDMS, however, resulted into high level of "cloudiness", which was not compatible with on-chip light microscopic observation of the multicellular spheroids within the microwells.

Next the cell-binding resistance of the Pluronic F127coated PDMS was determined *in vitro* in a long term cell culture assay. As expected, untreated PDMS samples had the highest amount of cell attached due to the hydrophobic property of PDMS. Using the untreated PDMS as reference, the cell repellence levels of Pluronic F127 treated PDMS at 3 days were calculated (Figure 2C). Cell resistance percentages were 81.6 ± 10%, 99.7 ± 1%, and 95.5 ± 3% for F127 physically adsorbed on PDMS, F127 swelled PDMS, and F127 adsorbed and then swelled PDMS, respectively. Compared with the untreated PDMS surface, all of the Pluronic F127 treated PDMS could significantly reduce cell attachment ($p < 0.05$). The fouling resistance of Pluronics coated PDMS is well-established and relate to the presence of the PEO chains extending in the liquid phase.[19,20] In agreement with the surface characterization study, the highest cell repellence level (99.7%) was observed for the direct swelling/deswelling method, whereas physical adsorption method resulted into 81.6% cell repellence. The direct swelling/deswelling was therefore selected for the modification of the PDMS microwells towards long term on-chip spheroid culture.

Multicellular Tumor Spheroid Formation Inside of the Concave Microwells. The formation of the multicellular spheroids within the concave microwells could be easily monitored using light microscopy. After being introduced into the macrowell containing the array of concave microwells, EMT-6 cells sedimented by gravity into each microwell by gently sliding down the funnel-shaped walls and in turn distributed evenly within the microwells. After the initial formation of loose cellular aggregates, which occurred within a few hours, dense and homogeneous multicellular spheroids were reliably obtained in the agarose microwells after 48 h. After optimization of the cell seeding density, the size of the spheroids was readily controlled by the dimension of microwells (Figure 3A−C). For example, at a cell seeding concentration of 1×10^5 cells/mL into the 600 μm microwells, the average size of the formed spheroids was 225 μm after 5 day's culture and 430 μm after 10 days of culture. Importantly, the micromachined mold approach enabled the design of microwells with high aspect ratios. In turn, spheroids were retained at the bottom of the microwells and not easily displaced during media manipulation, preventing the loss of the microtissues during culture medium changes and therefore

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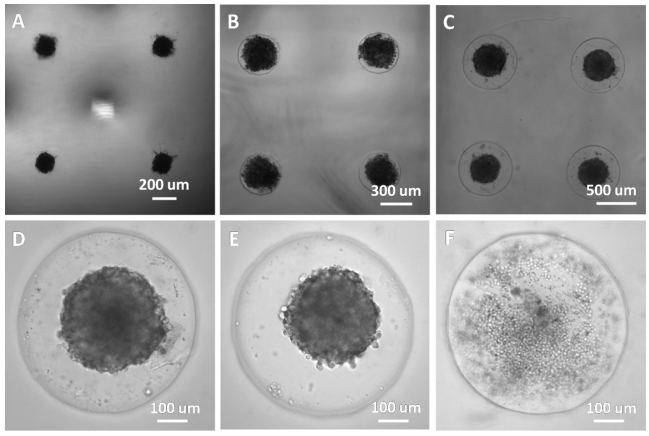

Figure 3. (A–C) Bright field microscopy of EMT-6 tumor spheroids formed in agarose microwells with diameters of 150, 350, or 600 μm. (D) High magnification of a spheroid formed in an agarose microwell after 10 days of culture. (E) EMT-6 cells cultured in Pluronic F127 swelled PDMS microwells formed homogeneous spheroids after 10 days. (F) EMT-6 cells cultured in Pluronic F127 adsorbed PDMS microwells for 10 days formed non-homogeneous cellular aggregates within the microwells.

facilitating long term on-chip culture. Compared with microwells with flat or cylindrical bottom prepared using standard photolithography, concave structures have been shown to foster cell–cell interactions and consequently the formation of high quality homogeneous multicellular spheroids. Cryosectioning of the microtissues using 25 um steps confirmed their expected spherical shapes. Homogeneous shape and size is an important feature in view of the use of multicellular spheroids in cancer research. To further investigate the quality of the tumor microtissues formed within the concave microwells, the formation of actin cytoskeleton structures within spheroids was investigated using phalloidin. Spheroids cultured for various amounts of time were fixed and stained with rhodamine phalloidin and imaged by laser confocal microscopy. All spheroids showed high cellular density, as shown in Figure 4. When the size of the spheroids was around 300 μm, corresponding for instance to 7 days of culture in the 600 μm microwells initially seeded with 2000 cells per microwell, cells were closely packed with uniformly distributed actin

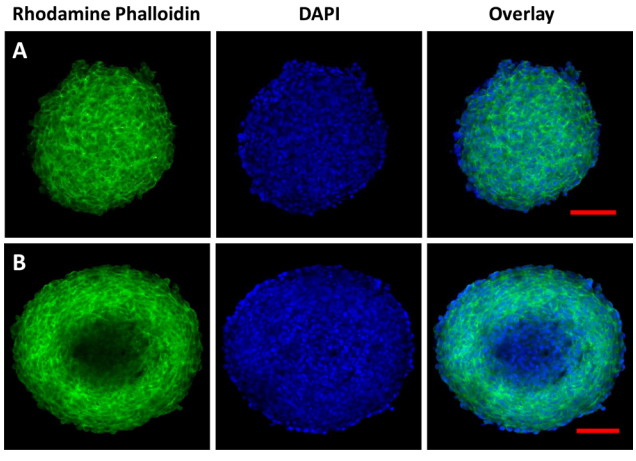

Figure 4. High resolution confocal imaging of spheroids stained by rhodamine phalloidin and DAPI: (A) spheroid with a size around 300 μm and (B) spheroid with a size above 400 μm. Scale bars are 100 μm.

filaments. This study confirmed the presence of tight cell–cell junctions and the associated cytoskeleton organization within the spheroid. All together with the uniform shape and size, these features confirmed the formation of high quality multicellular spheroids. For larger microtissues, however, typically above 400 μm in diameter, (obtained for instance after 10 days of culture in the 600 μm microwells initially seeded with 2000 cells per microwell), the intensity of the actin staining was reduced in the spheroids core, which might be linked to remodelling of the cytoskeleton during the process of formation of necrotic cores.

Next multicellular spheroids were grown inside PDMS microwells passivated using the swelling method with F127 Pluronics. No visible differences were observed for multicellular spheroids grown into the agarose or the F127-treated PDMS (Figure 3D, E). However, when the PDMS microwells were passivated with F127 physically adsorbed, no spheroids were obtained after 10 days of culture. The EMT-6 cells were found to attach on the PDMS surface with limited occurrence of cell–cell aggregation within the microwells as observed in the PDMS wells treated using the swelling method (Figure 3F). This observation confirmed that physically trapping F127 Pluronic molecules within the PDMS network enables long term cell-binding resistance, leading to excellent conditions for spheroid generation within the PDMS microwells. Importantly, although the use of high cell seeding density may hasten cellular aggregation and in turn spheroid formation within microfabricated microwells, the resulting tumor tissues may not have as high packing density as spheroids formed under long term culture.[3] Additionally, the cell–cell/cells and cells–ECM interactions may not be established completely after short term contact.[32]

Feasibility of On-Chip Drug Assay. Despite the attractive features of agarose microwells, including their inherent cell-binding resistance, long term use is somewhat cumbersome as agarose microwells needs to remain hydrated at all times. Loss of mechanical integrity of the hydrogel is also an issue in some instances. In addition, small molecules can readily diffuse within agarose, which may compromise their applicability for use in many biological assays. PDMS microwells were therefore used here to demonstrate the compatibility of the micromachined concave microwells with on-chip drug screening. Curcumin was used as a model anti-cancer drug in this feasibility study. Curcumin has multiple anti-cancer activities, reduced drug resistance, and excellent safety profile and is therefore an interesting low cost alternative to synthetic anti-cancer drugs.[33,34] The anti-cancer activity involves modulation of NF-κB, COX-2, AP-1, JNK, AKT, p53, and MAPK, suppression of the expression of cancer growth factor, and sensitizing chemotherapies and inhibition of multidrug resistance effects on both 2D cell culture and tumor spheroids.[34–36] The anti-cancer action of curcumin was investigated using mammary breast cancer EMT-6 spheroid formed within the PDMS concave microwells. Spheroids grown for 10 days in 300 um microwells were incubated with the drug for 24 h and then fixed and stained "on-chip" to determine cell viability using a live/dead assay. Importantly the whole assay could be conducted on-chip, without loss of microtissues during media manipulation and staining. A large number of dead cells were observed after incubation of the spheroids with 100 μM curcumin for 24 h. On the other hand, high cell viability was observed for spheroids treated with the DMSO vehicle control. Cell death percentages were calculated to be 6 $\pm$ 1% and 82 $\pm$

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8% for the control only and curcumin treated group, respectively (Figure 5). Although the live/dead assay could

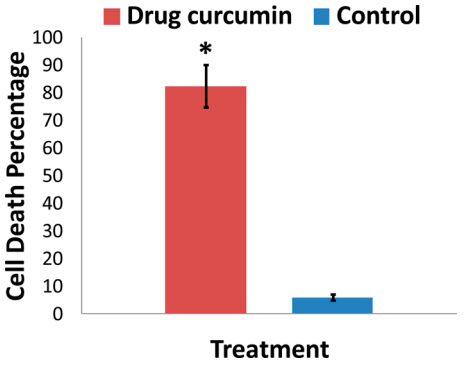

Figure 5. Cell viability obtained from on-chip Live/Dead assay after spheroids incubation with curcumin. Asterisks (*) indicate statistical significance compared to control at $p < 0.05$ using Student's t tests.

be reliably and easily performed within the microwells (including drug exposure, staining, and imaging), it is not readily compatible with high throughput studies. However, the characteristic features of the micromachined concave microwell arrays and most specifically the possibility to efficiently treat the microwells with drug or staining solutions without loss of microtissues, is important towards their application in on-chip bioassays using either microscopy based end-points such as the one conducted here or with metabolic assays such as the MTT. Future refinement of the microwell design will focus on achieving compatibility with liquid handling systems, which could be easily achieved through modifying the depth of the microwells "funnel-shaped" openings. A practical design could therefore be at the dimensions of standard tissue culture plates with for instance 1536 × 150 microwells per plates.

CONCLUSION

Multicellular tumor spheroids mimic closely the 3D cellular environment and recapitulate important pathophysiological gradients in *in vivo* tumors relevant to the establishment of the therapeutic activity of anti-cancer drugs. The development of efficient approaches to prepare high quality tumor spheroids and implement their use in standard biological screening assays is therefore of high importance. To this end, we demonstrate in this study that state-of-the-art CNC micromachining technology can be used to prepare metallic molds that can be replicated in agarose or PDMS to fabricate in one-step an array of deep concave microwells integrated in a primary macrowell. EMT-6 tumor cells seeded in the primary macrowell efficiently sedimented in the microwells and formed multicellular spheroids within 48 h. Further on-chip culture led to the formation of homogeneous spheroids of controllable size. To enable long term on-chip culture in PDMS microwells, a solvent-swelling method was successfully optimized to physically entrap the F127 Pluronic copolymers within the polydimethylsiloxane network and consequently increase its long term cell binding resistance. Finally, we show that the PDMS concave microwell platform is compatible with on-chip drug testing, demonstrating its relevance to the design of reliable high throughput spheroid-based drug assays.

ASSOCIATED CONTENT

Supporting Information

XPS data for anti-sticking PTFE-like coating are shown separately. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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