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Beyond the on/off chip trade-off: A reversibly sealed microfluidic platform for 3D tumor microtissue analysis

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ABSTRACT

Nowadays, microfluidic 3D cell culture is widely used to mimic complex microtissue and dynamic environment, performing more realistic *in vitro* assays for drug testing. Herein, we developed a novel microfluidic platform for tumor microtissue culture, drug response analysis and versatile microscopic characterization. By reversibly bonding the chip, we go beyond the on/off chip tradeoff, which allows us to perform both fluorescence and SEM characterization of tumor microtissues on a simple platform. The microfluidic chip consists of spherical microwells connected *via* microchannels, bonded through a magnetic system. Colorectal cancer HT-29 cells were cultured as spherical microtissues on chip and their growth kinetics monitored. The cytotoxic activity of Camptothecin was evaluated by *in situ* live/dead fluorescence staining and quantification of morphology parameters. Finally, we demonstrated the possibility to collect the 3D tumor microtissues and characterize their surface damaged by the drug using scanning electron microscopy. This reversibly sealed microfluidic platform thus enables to grow sets of 3D tumor microtissues in a controlled dynamic microenvinroment, and subsequently to retrieve the 3D tumor microtissues after chemotherapeutic treatment for in-depth analysis.

1. Introduction

Before the development of BioMEMS devices, animal experiments were long the main means to evaluate the efficacy of new drugs and therapeutic strategies [1]. Such experiments are however, associated with practical and ethical concerns [2]. In addition, efforts to develop new drugs based on data derived from animal models currently suffer from the unreliable transition of pre-clinical rodent data to clinical applications [3]. Due to these limitations, *in vitro* models became essential in different research areas, especially in cancer research [4]. 2D cell culture models were long the most used system for *in vitro* anticancer drug testing, until a wealth of research highlighted the limitations of these methods [5]. Interestingly, 3D cell culture provides important advantages over 2D ones for evaluation of drug candidates [6,7] including: (1) possibilities to create oxygen and nutrient gradients [8], (2) increased cell-to-cell interactions [9], (3) varying cell proliferation zones [10], and (4) the possibility to establish tumor-vascular interface models that closely resemble the natural pathological state of *in vivo* tumor microenvironment [11]. The impact of 3D microfluidic cell culture platforms for the testing of drugs and delivery systems are progressively growing in importance to curb the high cost of drug development and to accelerate drug discovery [12,13]. In recent years, many organ-on-a-chip start-ups were founded to develop microfluidic systems and tissue models to test drug efficacy and safety in advance of clinical testing [14]. In addition, the Food and Drug Administration officials recently announced a plan to evaluate tissue chips—also known as organs-on-chips—indicating that such chips could be used in future to test the effects of candidate pharmaceuticals and biologics as well as pathogens, biological hazards, cosmetics, and dietary supplements [15]. Thanks to these developments, animal research shortcomings can be overcome. In this context, microfluidic technologies enable the precise control of cellular microenvironment [16,17] and *ex vivo* tissue on chip [18], mimicking the fluid flow, interstitial flow pressure and shear stress, existing in a living organism.

To date, tumor spheroids represent the major 3D *in vitro* models that have been described over the past four decades for *in vitro* cancer research [19,20]. They are obtained after aggregation and compaction of cell suspension cultured in non-adherent conditions. Tumor spheroids are regarded as a versatile model for the study of cell proliferation and migration, the development of new therapies and drug screening, and the study of tumor-immune cell interactions [21,22]. Over the years, several methods were developed for the formation of spheroids: in

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suspension culture [23], with non-adherent surface [24], hanging drop [25] and microfluidic [26] methods. Compared to these existing methods, the microfluidic platforms offer many advantages for spheroids formation and analysis [27], such as high-throughput and low-cost for drug screening or physiological flow conditions for high fidelity micro-tissue models [28].{Hirschhaeuser, 2010, Multicellular tumor spheroids: an underestimated tool is catching up again;Vadivelu, 2017, Microfluidic Technology for the Generation of Cell Spheroids and Their Applications}

Ziolkowska and co-workers developed a microfluidic chip for long-term spheroid culture to evaluate anticancer drug activity [29]. Recently, many researchers have proposed microfluidic-based platforms for tumor spheroid development compatible with high-throughput commercial microplate testing, such as the SpheroChip system developed by Kwapiszewska et al. [30]. The advantage of microfluidics to enable anticancer drug evaluation has been recently demonstrated by others research groups too. In a recent work, Chen and co-authors presented a microfluidic chip to mimic the physiological microenvironment of solid tumor, studying tissue drug response at different concentrations of Doxorubicin [31]. Combined with the strong use in the bottom-up assembly of microtissues, microfluidic platforms have been proposed to develop micro-dissected tumor tissue on chip as an *ex vivo* method for drug testing [32]. Moreover, some recent works showed the potential of the image analysis to study the morphology parameters of spheroids subjected to drug treatments [33].

Although microfluidics offers various advantages, such as the reduction of sample volumes and minute control over the microenvironment, the irreversible bonding of the microchannels [4,17] prevents the spheroid retrieval for off-chip analysis. Several research groups have developed specific channel geometries to collect the spheroids by reversing [34] and increasing the flow rate [35]. This technique lacks the ability to retrieve the spheroids without cells damaging caused by high shear stress. According to extensive reviews from Vadivelu et al. [27] and Moshksayan et al. [36] about the state-of-the-art of microfluidic technology for the generation of cell spheroids, there is a dire need for reversibly sealed, reusable and modular microfluidic platforms for direct retrieval and off-chip characterization of tumor microtissues. Several techniques can be employed to further characterize a 3D tumor microtissue, especially to evaluate the efficacy of anticancer drugs [37]. For instance, electronic microscopy (scanning or transmission) is a useful complement to fluorescence imaging as it reveals details at the nanoscale level of 3D spheroids [38,39]. Furthermore, the loss of cell-cell physical interactions and the formation of holes in the spheroid surface due to cell death promoted, for instance, by an anticancer treatment can also be studied using this type of microscopy [40]. The development of temporary bonded and modular chips can help to recover the microtissue for a post-experiment off-chip analysis. In 2010, Kitamori and co-workers fabricated a modular microfluidic chip using a metal chip holder (screws) and surface chemistry modification, to collect the living cells cultured in the microchannel [41]. However, the modular chip obtained after multiple surface modifications was applied to form a 2D cell layer which is less relevant for antitumor drug testing in *in vitro* models. Chen et al also proposed a glass-PDMS-glass structure for the reversible bonding of microchannels, although the structure was not tested for the 3D cell culture [42].

Herein, we present an innovative modular microfluidic platform, easy to assemble, for 3D microtissue culture, drug response analysis and versatile microscopic characterization. The reversibly sealed chip is made of poly(methyl methacrylate) (PMMA) and polydimethylsiloxane (PDMS). The spin coating method, previously used to fabricate circular microchannels from square geometry [43], is here proposed to obtain round microwells from different geometries such as cylindrical microwells connected to microchannels. Unlike flow-recovery platforms, the choice of culture chamber geometry is somewhat arbitrary here. We chose round microwells as they are the most used microstructures for the spheroid formation and other applications, such as microlens array [44]. A system consisting of PMMA inserts for the magnets that helps to close the modular platform was used to reversibly bond the semi-circular microwells. The human colon adenocarcinoma HT-29 cell line was cultured on the developed resealable chip, as a model to optimize the formation of several sets of 3D tumor microtissues. Preliminary cytotoxicity experiments on tumor microtissues indicate that the proposed platform may allow *in vitro* drug testing assays. Furthermore, thanks to the reversible bonding process of the proposed platform, we collected the tumor microtissues for electron microscopy characterization. We compared the morphological details of the spheroids surface before and after drug treatment using the ImageJ software. Thus, the proposed platform allows a flexible combination of on-chip and off-chip characterization.

2. Materials and method

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, Camptothecin, Glutaraldehyde solution Grade I (25%) and Hexane puriss. p.a. (for the dilution of PDMS) 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 2 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, 400 and 200 µm (to mill the microwells) and 225 µm (to mill the microchannels), all these were purchased from Performance Micro tool (USA). Neodymium magnets, 5 × 5 mm (w × d) with a special epoxy coating were purchased from Magnets4you GmbH, Lohr a. Main (Germany). Tubing Silikon Peroxid/60 Shore ID 0.75 mm were obtained from IDEX Health & Science Gmbh (USA). Blunt Needle Plastic Hub 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). Tubing Tygon LMT-55 0.13 mm for peristaltic pump were purchased from Hydex Health & Science (Germany). 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 and Calcein AM were purchased from ThermoFisher Scientific Inc (USA).

2.2. Microfluidic chip fabrication and testing

The microwell-based chip, made of a hybrid combination of materials (PMMA-PDMS) was fabricated according to a previously described protocol [45]. The proposed method uses mechanical micromachining and PDMS thin layering techniques that are easy to use, reproducible, and inexpensive. To standardize the fabrication process to obtain round microwells from square geometry, we micromilled cylindrical microwells with different width–depth: 880–400, 400–200, and 200–100 µm. The microwell-based chip consisted of 2 lines of several microwells (880 µm diameter) connected by microchannels (225 µm diameter). The fabrication process of the reversibly sealed microwell-based microfluidic chip is outlined in Fig. 1a. The PMMA substrates were micromilled using a CNC micromilling machine, according to the layout illustrated in the supporting material (Fig. S3). The microwells and microchannels have an aspect ratio width/depth of 880/400 and 225/225 µm, respectively. We micromilled a dozen of holes (5 mm width and 2 mm depth), as inserts for the magnets. During micromilling, the spindle speed, feed speed and plunge rate per pass were respectively set to 10,000 rpm, 15 mm/s and 20 mm/s. After the micromachining step, round microwells and microchannels were prepared by spin coating