FROM 2D FLUIDIC ARRAY SCREENING TO 3D BACTERIAL CAPTURING STRUCTURES IN A POINT OF CARE SYSTEM FOR SEPSIS DIAGNOSIS

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ABSTRACT

A combined 2D microfluidic-microarray high throughput approach is reported to identify universal bacterial capturing ligands that can be tethered on the surface of 3D sponges fabricated by different methods for concentrating of bacterial targets in diagnosis devices. The developed platform allows for the first time the simultaneous monitoring of various ligands' affinities to different bacteria species in a dynamic condition *in vitro*. Moreover, it has been feasible to recognize the effect of steric hindrance on the function of capturing motifs through immobilizing spacer molecules with different lengths between the solid surface and ligands. 3D sponges and micropillars are modified with the most potent capturing molecule to assess their bacterial capturing in real blood samples. Next, the 3D structures are placed into a chip with an immense potential to recognize bacteria through imaging and fluorescence intensity concept.

INTRODUCTION

In Europe, more than 35% of patients in intensive care units suffer from sepsis, of which 27% die, leading not only to personal, but also an economic burden for the national health care systems. The cost of sepsis could be significantly reduced if faster early stage sensitive diagnostics were available by concentrating of pathogens from 5-10 ml blood sample to 10-20 µl test materials. The current gold standard for sepsis detection is conventional blood culture test that typically takes 14-48 h, having very low sensitivity [1,2]. Therefore, finding new universal capturing ligands that can rapidly attach to bacteria with high affinity is a key to improve the performance of the current diagnostic devices. This can prevent a costly and also unnecessarily antibiotics overtreatment for the patient if symptoms are due to other reasons rather than microbial infection. To achieve this goal, the first essential is a proper bacterial capturing motif to be assembled on diagnostic devices to let rapid diagnosis of early sepsis.

To recognize surface chemical structures with potent capturing efficiency, high-throughput screening (HTS) can be applied as the process of testing a large number of diverse chemical molecules in a simple, rapid, low cost, and high efficient manner, leading to a higher information harvest. In fact, high-throughput screening allows quick recognition of active compounds that can show specific biomolecular function. So far, scientists have applied this technique to discover new materials or biocompounds with specific capabilities, such as stem cell differentiation, immunophenotype modulation or bacterial resistance [3,4]. Using this method, here we elucidate the difference of various bio-ligands in terms of broad bacterial capturing competence to provide design guidelines for further progress in biosensing of sepsis.

There are different approaches to design and fabricate HTS platforms, of which micromilling assisted master mold design was used in this study. Micromilling technology is a very conventional, but convenient, cheap and precise tool in manufacturing a mold insert for replication of polymeric devices. Compared to the lithography process which usually needs several costly steps and resources [5], micromilling only needs three steps including computer-aided design (CAD), computer numerical control machining, and polishing, which is much faster than other available technologies and make this approach industrially friendly. Micromilling can be also easily used to fabricate microfluidic devices on polymer substrates for rapid testing and optimization applications. Moreover, since micromilling follows the design pattern to eliminate materials from a substrate, it can be used on different polymeric or metal based substrates, creating more possibilities in the formations of various microstructure patterns for various applications [5]. Therefore, this method was used in this study to create a master mold for the fabrication of a fluidic array that can be used for the HTS of various bacterial capturing ligands.

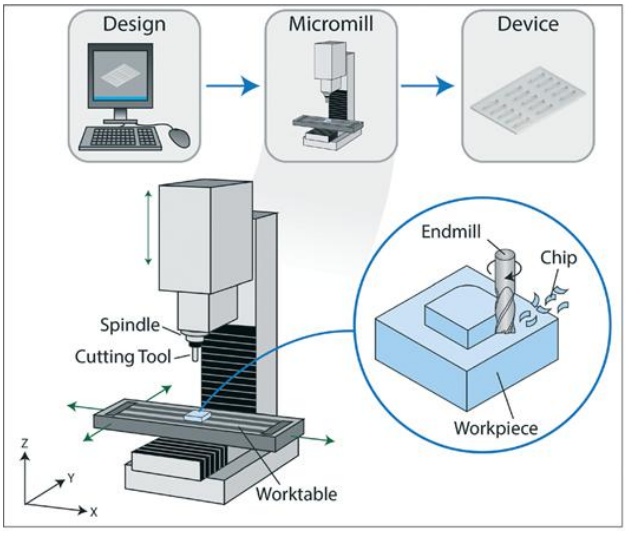

Figure 1. An illustrative representation of the basic components of a micromilling machine that uses CAD models to produce final devices. The machine consists of a worktable to provide plane for XY motion, a cutting tool for the removal of material from the workpiece, and a spindle that can hold and spin the cutting tool while providing motion along the Z-axis. Reprinted with permission from Ref [5].

In general, the main advantages of our suggested profiling technique are high throughput, parallelism,

miniaturization, speed and automation, all important factors needed to discover the most appropriate capturing ligand for developing a point-of-care bacterial identification chip. Micro-milling is used to prepare the mold for the fabrication of PDMS based fluidic array device as well as 3D micropillar structures used for capturing tests. The surface modification of the PDMS assembled on the bottom of the fluidic array for capturing is mediated by epoxide reaction that can results in very stable covalent binding of different bacterial capturing ligands to the solid surface of the platform.

EXPERIMENTAL METHODS

Design and fabrication of fluidic array

Poly(methyl methacrylate) (PMMA) is selected as the substrate material for micromilling due to its desirable properties, including high strength for hot embossing, its excellent optical property, low cost and facile substrate removal [16, 19–21;2 20-21;1]. AutoCAD Inventor was used to design the milling structure. The file was then transferred to CimatronE Micromilling software to let the micromilling machine read it and automatically setup the milling path. The file was loaded into the micromilling machine controlling system and the PMMA substrate with the dimension of 145 mm × 115 mm and a thickness of 9 mm was centralized on the micromilling stage. To mill the master mold for upper layer (channels) and the middle layer (wells) of the fluidic array, a 1500 µm milling bit was used for the overall milling process. Three steps of substrate removal was performed to achieve 2 mm substrate removal for the upper layer master mold and 2.5 mm substrate removal for the pillar formation as a mold for the fabrication wells for HTS screening. The cutting was performed 8,000 rpm with a feed rate of 100 mm/min. The total time required for fabrication of each mold insert was about 4-5 h.

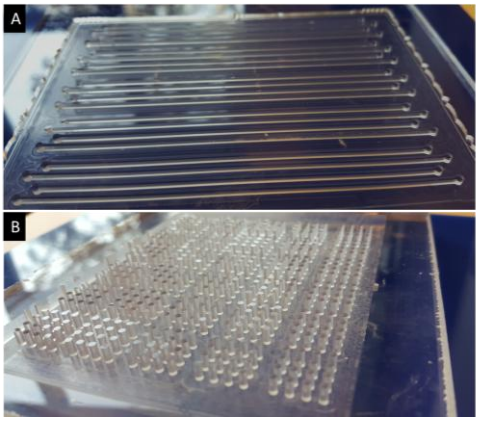

Figure 2. Master molds fabricated by micromilling technology for the preparation of the channels (A) and HTS wells (B). PDMS was used for casting to produce the HTS platform for bacterial capturing tests.

Next, the mixture of PDMS and curing agent (10:1) was poured onto the molds and the samples were then transferred within a vacuum desiccator at room temperature for 3 days to let the PDMS get cured and become hard while being degassed to remove all air bubbles. Afterwards, cured platforms were peeled off, treated by plasma oxygen and then the upper channel platform was placed on the PDMS made wells under pressure for 1 day to crosslink them and fabricate the fluidic array.

Surface modification and characterization of PDMS

To functionalize the surface of the PDMS structures with epoxide group, surface oxygen plasma treatment was first performed on all PDMS samples. To do so, the PDMS surface was first cleaned with isopropanol, dried, and then treated with oxygen plasma for 2 min under vacuum using plasma cleaner. Right after surface hydroxylation, the samples were immersed in a freshly prepared 4% v/v solution of (3-glycidyloxypropyl)trimethoxysilane in ethanol, and incubated for 150 min at RT. The samples were then washed gently with ethanol, cured at 80 °C for 1 h, and sonicated in ethanol for 10 s to remove the physically adsorbed and unbound (3-glycidyloxypropyl)trimethoxysilane molecules. Rinsing with ethanol was again performed in the next step and then the samples were blown dry with a nitrogen stream. The obtained epoxide functionalized PDMS structures were then stored in a sealed container at room temperature until use.

To confirm the epoxide modification of the PDMS, the samples were treated for 48 h with albumin-FITC dissolved at a concentration of 1mg/mL in 50 mM borate buffer pH 8. The fluorescence of the PDMS was then monitored using a scanner as a consequence of epoxide reaction with the amine groups of the protein.

To do surface modification with peptide, the 2D epoxide modified PDMS was placed on the bottom of a small baker and then the cecropin peptide with the concentration of 10 µg/mL was added and left at 4˚C for 48 h under slight agitation. Next, the surface of the PDMS was washed with PBS buffer (pH 7.4) before locating it as capturing surface on the bottom of the designed fluidic array (Figure 2).

Bacterial capturing studies

Four species of bacteria, including two gram positive (staphylococcus aureus and enterococcus) and two gram negative (salmonella typhimurium and E.coli) were injected into the fluidic array and the capturing surface of each well was exposed to the bacteria for 30 min at 37 ˚C before washing with PBS (pH 7.4) for two times and then fixing the captured bacteria by paraformaldehyde 4% for 1 h. Next the samples were coated by gold sputter and the bacterial capturing was monitored under scanning electron microscopy (SEM) by counting the number of bacteria per surface area.

Construction of 3D PDMS structures

For 3D flexible sponge formation, sugar cubes, compressed NaCL, and compressed ground NaCl were used as template for PDMS casting. In sugar cube mediated method, the mixture of silicone elastomer and curing agent were drop-casted on the sugar cube and infiltrated into the porous structure by capillary force under vacuum for 1 h. The samples were then placed for 24 h at 50 °C, and later, the present on the surface of the sugar cube was wiped off till sugar surface exposed. Afterwards, the sugars were dissolved in deionized water

and washed away under sonication bath for 4 h before washing with ethanol and drying the sponges at 50 °C for 6 h.

As for NaCL mediated sponge formation, both non-ground and ground NaCL (the grain sizes of the non-ground NaCL powders were reduced by grinding for 15 min using a pestle and mortar) were moisturized and then tightly packed within 50 mL syringes. Then the syringes were located within oven for 24 h at 50 °C to let the salt crystals dry by water evaporation. In the next step, the mixture of PDMS and curing agent, with same ration as mentioned above, was poured onto the Syringe while the syringe tip was connected to a vaccum tube to help sacking PDMS within the salt microstructure template while simultaneously removing air from the intra-spaces of the packed NaCL. The samples were then transferred within a vacuum desiccator for 45 min to degass and remove all air bobbles within the template before baking the sample in the oven at 50 °C for 24 h. In the last step, the samples were cut into small desirable pieces with appropriate thickness and immersed in deionized water for 22 h under agitation and 1 h in bath sonicator to remove all salt powders. Micropillars were also fabcricated by PDMS using a master mold micromilled on PMMA.

X-ray Computed Tomography Scans

X-ray computed tomography scans were conducted on the CGOLSF membrane with a lab-based Xradia Versa XRM-410 instrument using a W reflection target at a power of 10 W. The scan was performed at a voltage of 150 kV, with an HE03 filter (Xradia), 10× optical magnification, binning 4, an exposure time of 4 s and a total of 1601 projections over a 360° rotation, resulting in a pixel resolution of 3.8 µm. The recorded data were reconstructed using a Feldkamp-Davis-Kress reconstruction with a smooth filter (0.5 kernel size) and beam hardening constant of 0.3. After reconstruction, segmentation was performed, separating membrane solids and pores by using global thresholds with the software Avizo fire 8.0 (for the total 3D pore volume). Additional segmentations were performed to estimate errors in the quantitative analysis.

Water contact angle (WCA) measurements

The contact angle (CA), θ, was measured at the surface of the 2D PDMS and plasma treated 3D sponges as well as internal compartment of the 3D sponge after cutting the sponges from the middle using a contact angle instrument (model) at 25 ± 2 °C. A water droplet was gently dropped on the surface of the samples using a syringe. All of the droplets were released from the syringe needle by touching the surface of the samples to minimize the inconsistency between each measurement. The angle between the tangent line and the surface of the samples was measured right after the release of each droplet onto the surface.

SEM imaging of 3D structures

To investigate the impact of different preparation method on the morphology and pore size of the 3D sponges, SEM imaging was performed for all the samples by a Quanta 250 SEM (FEG, USA). For this purpose, all different types of sponges were separately embedded on the SEM holder using double sided carbon adhesive tape. The samples were then left in vacuum for 25 h to ensure their dryness. Next, the surface of the samples was sputtered by Au in a high vacuum evaporator (Q150TS, Quorum Technologies, UK) before imaging.

RESULTS AND DISCUSSION

Fluidic array fabrication and characterization

PDMS based fluidic array was successfully fabricated and tubing was conducted without observing any leakage from the channels. The epoxide surface modification of the PDMS capturing surfaces (Figure 3C) was also confirmed by albumin-FITC in which the fluorescence signal could be detected (Figure 3D).

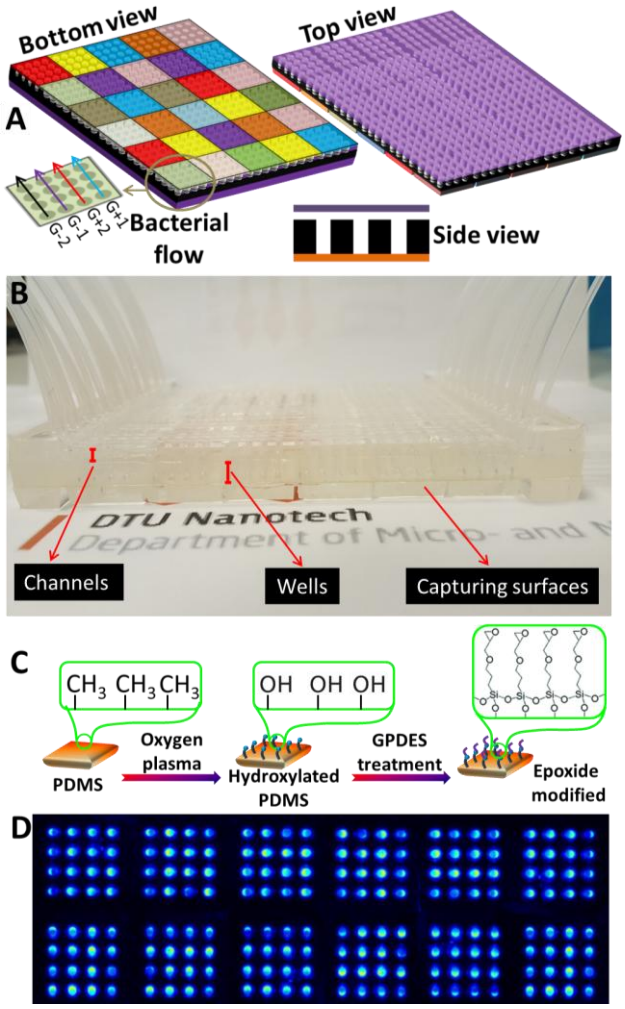

Figure 3: Fluidic array for high throughput profiling of universal bacterial capturing ligands. The device consists of 30 different surface chemistries on the bottom side as shown with different colors, a middle part with 480 holes shown in black (16 holes for each surface chemistry) and an upper part that connect holes of each line for bacterial flow (A). Fabricated PDMS fluidic arrays for bacterial capturing studies (B). Epoxide modification and its reaction with albumin-FITC (C and D).

To further confirm the surface modification of the 2D

PDMS used on the bottom of the fluidic array as capturing surfaces, the contact angle of the PDMS was measured in each step of surface modification. The changes in contact angle, shown in Table 1 prove the successful surface modification in each step.

Table 1: Contact angle of PDMS before and after surface modification with cecropin peptide. Light and dark gray colors are representative of 2D and 3D surfaces, respectively.

Unmodified	Plasma treated	Epoxy modified	Cecropin modified
110.6±1.9	57.3±2.1	99.8±0.4	62.2±0.7
140.7±0.2	49.4±0.8	111.5±0.6	57.4±0.3

The successful conjugation of peptide to the surface of the 2D PDMS was confirmed by the reaction of amine groups in peptide with FITC-NHS and detection of fluorescence signal (Figure 4A). The 2D PDMS without cecropin on the surface showed no fluorescence signal. The bacterial capturing test showed that cecropin can more efficiently bind to the gram negative bacteria.

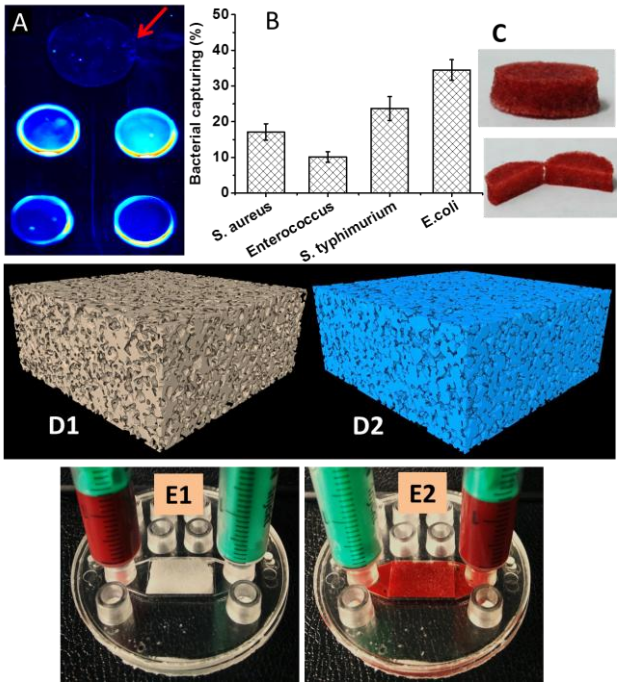

Figure 4: Confirmation of peptide binding to the epoxide groups (A) through FITC-NHS reaction with the amine groups of the peptide. The control is shown with red arrow. The percentage of bacterial capturing of cecropin modified surfaces in the fluidic array device (B). The interconnectivity of the sponge tested by real blood samples (C), showing blood can reach all areas within the sponge. X-Ray tomography analysis of salt templated sponge (D1) and its corresponding pore area (D2). Testing sponge insertion within the fluidic device and blood flow through the device (E1 and E2).

The interconnectivity and X-ray topography test showed high porosity for all 3D structures and the absence of voids in NaCl templated sponges (Figure 5C, D1 and D2). The sponges did not show a significant resistant to blood flow and they could be easily inserted within a chip prepared by injection moulding method (Figure 4E). 3D micropillars and NaCl templated PDMS were successfully fabricated by PDMS casting on the mold (Figure 5).

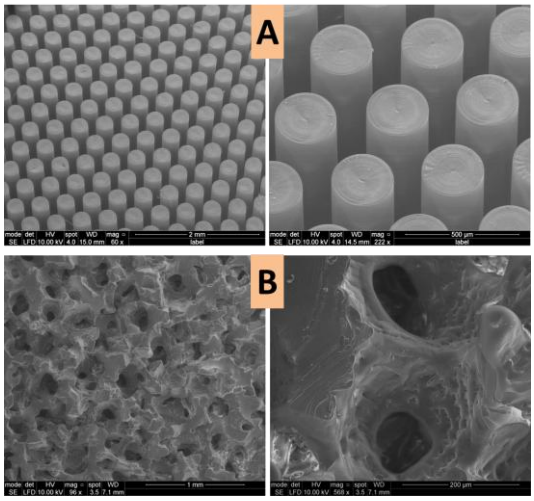

Figure 5: The surface morphology of the 3D PDMS micropillar (A) and NaCL templated sponge (B), both developed to be tested in the bacterial capturing device after conjugating capturing ligand on their surface.

CONCLUSION

Effects of bacterial capturing ligand on the sample concentration were investigated. A fluidic array was successfully designed for this purpose, elucidating the possibility of high throughput screening of capturing ligands. The ligands were conjugated on the bottom surface of the array to screen the bacterial absorption. Cecropin peptide showed more capability to capture gram negative bacteria than gram positive. The 3D PDMS based structures were also fabricated to be used in the diagnostic device for bacterial capturing.

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