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Nano-biofilm Arrays as a Novel Universal Platform for Microscale Microbial Culture and High-Throughput Downstream Applications

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Abstract

Biofilms are the predominant mode of microbial growth and it is now fully accepted that a majority of infections in humans are associated with a biofilm etiology. Biofilms are defined as attached and structured microbial communities surrounded by a protective exopolymeric matrix. Importantly, sessile microorganisms growing within a biofilm are highly resistant to antimicrobial agents. Thus, there is an urgent need to develop new and improved anti-biofilm therapies. Unfortunately, most of the current techniques for *in vitro* biofilm formation are not compatible with high throughput screening techniques that can speed up discovery of new drugs with anti-biofilm activity. To try to overcome this major impediment, our group has developed a novel technique consisting of micro-scale culture of microbial biofilms on a microarray platform. Using this technique, hundreds to thousands of microbial biofilms, each with a volume of approximately 30–50 nanolitres, can be simultaneously formed on a standard microscope slide. Despite more than three orders of magnitude of miniaturization over conventional biofilms, these nanobiofilms display similar growth, structural and phenotypic properties, including antibiotic drug resistance. These nanobiofilm chips are amenable to automation, drastically reducing assay volume and costs. This technique platform allows for true high-throughput screening in search for new anti-biofilm drugs.

Keywords

biofilms; microarray; biofilm chip; microscale culture; high throughput screening; drug development

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise..

1. INTRODUCTION

The majority of microbes in their natural habitats persist attached to surfaces within a structured biofilm ecosystem and not as free-floating (planktonic) organisms [1–2]. Although they were first described by Antonie van Leeuwenhoek, the theory describing the biofilm process was not developed until late in the 20th century [1]. For the purpose of this review, we define biofilms as attached and highly organized microbial communities encased within a matrix of self-produced exopolymeric materials. In the last three decades there has been an increased recognition of the role that biofilms play in medicine, and now it is commonly accepted that 60–80% of all human microbial infections involve biofilm formation [3–4]. In a patient biofilms can form on both biotic (*i.e.*, skin, tissue) and abiotic (*i.e.*, implants, catheters and other indwelling devices) surfaces [5–6]. Once formed, these biofilms can initiate or prolong infections by providing a safe haven from which cells can invade local tissue, seed new infection sites, and also lead to failure of implanted medical devices [5–6]. Most importantly, sessile cells within the biofilms display high level of resistance against most clinically-used antibiotics, often leading to treatment failures and possibly death [7]. Overall, microbial biofilm formation carries important negative clinical repercussions, adversely impacting the health of an ever increasing number of patients, also with mounting economic costs to our health care systems [8]. Undoubtedly there is a dire and unmet need for novel antibiotics capable of treating biofilm-associated infections [9–11].

Remarkably, with few exceptions, techniques for microbial cell culture have changed relatively little in the last two centuries since the time of Pasteur and Koch [12–14]. Most microbiology laboratories today still employ traditional culture techniques using Petri dishes, test tubes, and Erlenmeyer flasks; typically with volumes ranging from liters down to milliliters. In regards to biofilms, most techniques for their formation are still relatively burdensome, often requiring expert handling, long processing times and the use of specialized equipment; and allow for the formation of relatively few equivalent biofilms at the same time. Perhaps the most significant improvement was made with the introduction of 96-well microtiter plates, which offers a relatively simple and inexpensive alternative for biofilm formation and increased the culture densities by 10- to 100- fold compared with that of previous biofilm-forming techniques [13]. However, most of these current techniques for *in vitro* biofilm formation are not compatible with modern methodologies for drug discovery that are dominated by high throughput screening (HTS) and its “hunger for speed”, thus severely compromising our ability to identify new molecules that could be further develop as novel anti-biofilm therapeutics.

Here we describe our previous work on the development of a new nano-biofilm cell culture platform that allows for the formation of hundreds to thousands equivalent biofilm at micro-scale level and is fully compatible with ultra-high-throughput drug discovery.

2. High-Throughput *Candida albicans* Biofilm Chip

For our initial attempts to develop this new technology platform we used *Candida albicans* as a model organism. This opportunistic pathogenic fungus the main causative agent of

candidiasis, now the third most frequent nosocomial infection with unacceptably high mortality rates [15–17]. Our group and others have previously reported on the ability of *C. albicans* to form biofilms on a variety of surfaces and indeed, biofilm formation is associated with a majority of manifestations of candidiasis, from oral infection to catheter-related candidemia and invasive disease [18–19]. The antifungal arsenal is exceedingly short and, not surprisingly, *C. albicans* biofilms are resistant to most clinically-used antifungal drugs [20–21]. Thus, there is an urgent need for the development of novel antifungal agents, particularly those that are active against fungal biofilms [21].

Our initial goal was to develop a high density chip of spatially addressable three-dimensional biofilms, which according to our criteria and ultimate goals should meet certain conditions, including: *i*) a single chip should contain hundreds-to-thousands of spatially distinct biofilm cultures; *ii*) despite miniaturization the individual biofilms should display phenotypic characteristics similar to those of conventional biofilms (*i.e.* growth, morphology, structure and drug resistance); *iii*) it should not dry easily to allow for prolonged incubation times if required; *iv*) it should attach firmly to the substrate and should be able to withstand multiple washings; and finally *v*) it should be fully compatible with standard microarray technology and analysis with a standard microarray scanner, as these pieces of equipment may be widely available in many laboratories or institutions [22]. Our initial attempts consisted of a series of proof-of-concept experiments using factorial design for the simultaneous assessment of multiple parameters of biofilm formation, including culture conditions (inocula, growth media), slide surface modification, and encapsulation procedures [22]. After extensive optimization, a cellular microarray prototype consisting of nanoliter-scale cultures of *C. albicans* biofilms was prepared by mixing a suspension of yeast cells, microbiological media and a hydrogel solution, which were then deposited onto the surface of modified microscope glass slides using a robotic microarray spotter [22], as described below in more detail.

Borosilicate (glass) slides were first modified by chemical treatment in order to provide a hydrophobic surface, which represents a major requirement for the deposition of spatially isolated liquid spots in the nanoliter range onto the surface of the microscope slide. To this effect we first pretreated the microscope slides with 3-aminopropyltriethoxysilane (APTES), and subsequently with polystyrene-co-maleic anhydride (PSMA). The styrene-co-maleic anhydride molecules zip together forming a mono-layer made of two molecules in cross section, which results in enhancement of the substrate hydrophobicity [22]. Importantly, at each step, the surface was analyzed by Fourier transform infrared (FTIR) spectroscopy to confirm and determine the molecular structure on the modified surface [23]. The PSMA also provided sufficient functionality for subsequent binding to an encapsulating matrix, consisting of a collagen or an alginate hydrogel matrix used in subsequent steps of chip formation. These natural hydrogels have been widely used for cell-encapsulation studies because of their favorable gelation and biomimetic properties [24], and the use of an encapsulating matrix was also deemed necessary in order to form robust biofilms capable of resisting multiple washing cycles (Figure 1A).

After optimization of all different parameters, the high density arrays were printed using a robotic microarrayer [22–23]. For preparation of inocula for printing and nano-biofilm

formation in the microarray, cells were harvested from overnight cultures, washed and resuspended at a given cell density in appropriate microbiological medium. The inoculum was then mixed with the encapsulating matrix of choice. This final suspension containing microbial cells, media, and matrix was printed, typically at 30 nL per spot, on the functionalized PSMA-coated glass slides by using a noncontact microarray spotter with wide orifice ceramic tips to allow for printing of cells. After robotic printing, the slides were placed in a humidified hybridization cassette to prevent evaporation of spots and incubated at 37°C for approximately 18 – 24 h to allow for biofilm growth and maturation, yielding the final nano-biofilm microarray (Figure 1B). If done properly, the individual spots should remain hemispherical and attached robustly to the surface, supporting true biofilm growth [22–23]. In its full potential, a typical chip can encompass up to 2,000 individual spots of 30 – 50 nL volume of identical nano-biofilms, and multiple chips can be printed simultaneously [22–23]. Of interest, we observed that the nano-biofilms were best formed over a relatively narrow range of media concentration and inoculum density.

Finally, the extent of biofilm formation on the microarray can be monitored by staining with appropriate fluorescent dyes, and this fluorescent readout also improves on conventional well plate-based spectroscopic methods [22–23]. It is important to use dyes whose excitation and emission spectra are compatible with the filters installed in the microarray scanner. If the goal is to assess antibiotic activity, a viability dye should be used (*i.e.* FUN1). Staining can be performed by immersion of the entire microarray slide in a staining jar containing a dilution of the dye followed by washings using a simple dunk-and-rinse procedure to remove excess stain. After air-drying, the chip is ready to be read using a microarray scanner. The resulting microarray scanner images can be archived and analyzed using appropriate software. Importantly, we have demonstrated using specific dyes that levels of fluorescence correlate linearly with cell number over the range of interest[22] (Figure 1D). We also found that the fluorescent intensities from spots that are seeded at same initial cell density were statistically indistinguishable indicating uniform distribution of biofilms at different locations on the microarray [22]. Thus, all nano- biofilms formed in the same chip are equivalent to each other, which is imperative for its use in large scale high throughput/high content screening applications.

3. Characterization of phenotypic properties of nanoliter-scale biofilms

As mentioned above, each individual nano-biofilm formed in these chips is approximately 30 – 50 nL in volume, which represents a 2000 – 3000 fold reduction as compared to conventional biofilms formed for example in microtiter plates [13, 22–23]. Thus, it was important to demonstrate that, despite miniaturization, the resulting nano-biofilms maintained phenotypic characteristics typical of a true biofilm and fully consistent with a biofilm mode of growth.

We used Scanning electron microscopy (SEM) and Confocal laser scanning microscopy (CLSM) to examine the morphological and architectural characteristics of nano-biofilms formed in the chip [22–23]. In the case of our original chip using *C. albicans*, SEM indicated that morphologically these nano-biofilms consisted of a mixture of yeast cells and hyphae, similar to what has previously been described for “macroscopic” biofilms [22]. SEM also

allowed for the visualization of how microbial cells are embedded within the encapsulating matrix (Figure 1C). Contrary to SEM, the non-destructive nature of CLSM allows for the visualization of biofilms in its native state, and we used CLSM to routinely monitor for morphology, spatial distribution, and the overall architecture of the nanoliter-scale biofilms [22–23] (Figure 2). Results indicated that biofilms formed in the chip exhibited a high degree of spatial heterogeneity, with regions of metabolically active cells interspersed within the encapsulating matrix, and an overall thickness of the biofilm estimated to be approximately 50 μm (Figure 2A). Thus, from the point of view of their morphological, structural and architectural properties the nano-biofilms within the chip are similar to biofilms formed using standard methodologies [25–26].

It is well established that the formation and maturation of biofilms are associated with high levels of resistance to antibiotics[27]. In the case of *C. albicans*, biofilms are notoriously resistant to azole antifungal agents (up to 1,000 times more resistant than their planktonic counterparts)[27–28]. Thus, after having demonstrated the morphological and growth characteristics of nanoliter-scale biofilms on the microarray, it was also important to ascertain that these miniaturized biofilms display resistant properties comparable to those of macroscopic biofilms grown in 96-well plates (representing the current standard in the field), which is of critical importance if the chips are going to be used in HTS techniques in search for new drugs with activity against biofilms. Using the robotic arrayer, drugs were spotted on top of the mature pre-formed biofilms on the chip, incubated for an additional 24 h period, followed by addition of the FUN 1 metabolic dye [22–23]. It was demonstrated that nano-biofilms display similar profiles of susceptibility and resistance against different types of clinically-used antifungals (amphotericin B, fluconazole and caspofungin) as those formed using the widely used microtiter plate model [22–23] (Figure 1E). A caveat here is that the choice of encapsulating matrix may lead to differential susceptibility to different antibiotics depending on the interaction or lack thereof between the matrices and the drugs [29]. For example, consistent with its high levels of binding to serum, we demonstrated lower activity of caspofungin against collagen-encapsulated *C. albicans* biofilms [29].

4. Expansion to bacterial and polymicrobial biofilms

Although our initial development of the nano-biofilm chip used the fungus *C. albicans*, the technology is flexible and it was our intention to expand it as a platform for universal microbial culture, to provide proof of concept that different microorganisms can be grown using this technique. Thus, in subsequent experiments we demonstrated its versatility by successfully developing nano-biofilms chips of both *Staphylococcus aureus*, a Gram-positive bacterium and leading cause of nosocomial infections often associated with biofilm formation, and *Pseudomonas aeruginosa*, a Gram-negative bacterium well known for causing biofilm infections in cystic fibrosis patients and other settings [30]. As in the case of *C. albicans*, the resulting miniaturized bacterial biofilms demonstrated characteristics similar to those displayed by conventionally formed macroscopic biofilms for the two species, including architectural features (Figure 2), synthesis of biofilm matrices, and high levels of resistance to antibiotics [30]. In an extension of these studies, most recently we demonstrated the applicability of a similar technique for culturing community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA), and perform antibiotic

susceptibility testing against clinical isolates of CA-MRSA, which required significantly smaller sample volumes compared to conventional methods and resulted in much faster results [31].

Moreover, there is an increased recognition of the fact that most infections are polymicrobial in nature, and among these *S. aureus*, *C. albicans*, and *P. aeruginosa* are among the most common etiological agents contributing to high morbidity and mortality rates associated with these difficult to treat polymicrobial infections [32–33]. Thus, we further expanded our technology for the formation of polymicrobial biofilms, including mixed bacterial *S. aureus*/*P. aeruginosa* biofilms as well mixed fungal/bacterial biofilms of *S. aureus* and *C. albicans* (Figure 2C) at the nanoliter-scale level [30]. These results support the fact that this technology can become a universal platform for microbial culture at the nanoliter-scale level.

CONCLUSION

Overall the described technology platform for the formation of microbial nano-biofilm chips is highly versatile, and can be used to generate fungal, bacterial, as well as polymicrobial biofilms, thereby constituting a universal platform for microbial cell culture at the nanoliter-scale level. The technique is compatible with standard microarray technology and equipment. By virtue of its automation and miniaturization, it dramatically reduces costs and cuts reagents use. From an evolutionary standpoint, this technology platform heralds a new era in ultra-high throughput screening assays for the identification of novel compounds with anti-biofilm activity, which are urgently needed; and has the potential for changing the face of the antibiotic drug discovery process. Besides HTS, this technology holds promise for other high-content assays and downstream applications, including to name a few: high-throughput phenotypic testing, the screening of large collections of mutant libraries, large-scale screening of peptides, aptamers, and nanoparticles; studies on the microbiome and metagenome; and applicability to studies on biofilm-biomaterial interactions. It is our hope that the application of this technology will lead to the discovery of the next class of wonder drug, to the development novel coatings of biomaterials such as catheters and stents to prevent microbial adhesion and biofilm formation, as well as to the next generation of techniques for accelerated antibiotic susceptibility testing.

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Figure 1.

A) A schematic of a single nano-biofilm spot on a modified glass slide showing functionalization of the surface. B) Microarray scanner image of nano-biofilm microarray with 1,200 spots of identical nano-biofilms of *C. albicans* of 400-μm diameter each. C) A series of SEM images at increasing magnification (32 x, 200 x and 1.4K x) of *C. albicans* nano-films formed in the chip after 24 h incubation showing morphological features of cells within the biofilm with the presence of hyphae embedded within the hydrogel. D) Validation of the fungal biofilm chip for high-throughput analyses: the graph shows the linear correlation between number of cells and fluorescence intensities of spots obtained after reading with the microarray scanner. E) Dose-response curves showing inhibition of biofilm formation by amphotericin B treatment. Adapted from references [22] and [23]. Please refer to the original articles for experimental details.

Figure 2. Confocal Laser Scanning Microscopy images of nano-biofilms of *C. albicans* stained with FUN1 (A), *P. aeruginosa* stained with SYTO-9 and SYPRO Ruby (B) and mixed *C. albicans*/*S. aureus* stained with FUN-1 and concanavalin A (C). Adapted from references [22] and [30]. Please refer to the original articles for experimental details.