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Droplet Microarray as a Powerful Platform for Seeking New Antibiotics Against Multidrug-Resistant Bacteria

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Multidrug-resistant (MDR) bacteria is a severe threat to public health. Therefore, it is urgent to establish effective screening systems for identifying novel antibacterial compounds. In this study, a highly miniaturized droplet microarray (DMA) based high-throughput screening system is established to screen over 2000 compounds for their antimicrobial properties against carbapenem-resistant *Klebsiella pneumoniae* and methicillin resistant *Staphylococcus aureus* (MRSA). The DMA consists of an array of hydrophilic spots divided by superhydrophobic borders. Due to the differences in the surface wettability between the spots and the borders, arrays of hundreds of nanoliter-sized droplets containing bacteria and different drugs can be generated for screening applications. A simple colorimetric viability readout utilizing a conventional photo scanner is developed for fast single-step detection of the inhibitory effect of the compounds on bacterial growth on the whole array. Six hit compounds, including coumarins and structurally simplified estrogen analogs are identified in the primary screening and validated with minimum inhibition concentration assay for their antibacterial effect. This study demonstrates that the DMA-based high-throughput screening system enables the identification of potential antibiotics from novel synthetic compound libraries, offering opportunities for development of new treatments against multidrug-resistant bacteria.

1. Introduction

Antimicrobial-resistant infections pose a severe threat to human health. There are nearly 700 000 people in Europe and more than 2 million people in North America suffering from antimicrobial-resistant infections every year.[1] Multidrug-resistant bacteria, including the "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp*) caused over 15% of nosocomial infections, which are difficult to cure by most of the traditional antibiotics.[2] To combat multidrug-resistant bacteria, efforts should be made to facilitate laboratories to develop new antibiotics. High throughput screening (HTS) is a method enabling the selection of drug candidates from large compound libraries. Current widespread methods for antibiotic screening such as the disk diffusion assay and the broth dilution test, are mostly based on microplates and agar plates.[3] Using these platforms for

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conducting HTS requires automation and large number of compounds, leading to high costs hindering ordinary laboratories to perform screening of large compound libraries and to identify novel antimicrobial compounds.

To reduce the cost and improve the efficiency of HTS, the miniaturization of screening platforms has attracted the attention of scientific community in the last years. For example, high-density microplates enable few microliters of working solution per well.[4] M. de Vos and co-workers fabricated a miniaturized microbial culture chip by etching acrylic polymers coated on the surface of porous ceramic. This chip containing up to one million growth compartments enables screenings of fluorescent microcolonies and galactosidase-producing microcolony.[5] Various microfluidic platforms, in particular droplet microfluidics, present great potential for HTS of bacteria. For example, Smith and co-workers reported a microfluidic-based pico-droplet platform enabling screening of bacteria in picoliter droplets by measuring of optical density to select antibiotic-resistant strains.[6] Finally, high-density peptide arrays (10^6 spots cm^{-2}), based on on-chip solid-phase synthesis, are used for on-chip screenings for antimicrobial peptides.[7] Despite the progress made in development of miniaturized solutions for high throughput bacterial screenings, limitations of the above platforms need to be overcome or improved, e.g., their compatibility with lab equipment, issues caused by small working volumes such as evaporation and liquid adhesion, and practical approaches to transfer libraries to platform systems.

Along with the trend of miniaturization of bacterial screening, in this study we utilized the droplet microarray (DMA) platform for high throughput screening for antimicrobial compounds with MDR bacteria in nanoliter droplets. DMA is based on hydrophilic-superhydrophobic patterning. Due to the extreme difference in wettability of hydrophilic and superhydrophobic regions, it is possible to form arrays of hundreds aqueous droplets of 150 nL volumes, which serve as miniaturized compartments for growing and testing bacteria. DMA is a representative example of applications in cell arrays of superwettability-based patterned surfaces.[8] In our previous study, a sandwiching method has been applied to add antibiotics into droplets containing GFP expressing bacteria simultaneously across the whole DMA following by estimation of bacteria growth by microscopy.[9] In that study we have successfully identified the drug resistance of *P. aeruginosa* 49 by a screening of a small library containing 18 antibiotics.[10]

In current study, we have screened a pre-printed on-chip libraries of over 2000 compounds for their ability to inhibit the growth of drug-resistant bacteria. The tested library consists of compounds from the DFG Core Facility Molecule Archive (https://risources.dfg.de/detail/RI_00502_de.html). The composition of the academic compound library is highly diverse and contains different natural products and natural product-like compounds, such as structurally distinct heterocycles and steroids/ steroid-type compounds and peptoids deriving from different synthetic projects. Furthermore, the library contains precursors and end products from research projects focusing on applications in materials sciences, such as phosphines, porphyrins, or quinazoline derivatives.

The following MDR bacteria were used in the screening in this study: *K. pneumoniae* ATCC BAA-2146, and *S. aureus* A1 *mec*A^r. *K. pneumoniae* ATCC BAA-2146 is Gram-negative bacterium and belongs to a family of carbapenem-resistant Enterobacteriaceae (CRE), which is referred to as an "urgent threat" to public health in the report of *Antibiotic Resistance Threats in The United States 2019*. Double or triple antibiotic combinations are required to treat infections caused by *K. pneumoniae*.[11] *S. aureus* A1 *mec*A^r is a Gram-positive bacterium, which is methicillin-resistant (MRSA), causing potential fatal diseases including bloodstream infections, pneumonia, or surgical site infections.[12]

In addition, we have applied a simple single-step read-out method, based on tetrazolium salt WST-8 colorimetric viability dye, where we used a photoscanner to acquire a digital image of the whole array containing 588 experiments.[13] In addition to finding novel substances with antimicrobial potential against MDR bacteria, we have established and validated the miniaturized platform for high throughput screening of bacteria, which is compatible with large compound libraries (due to its 2D array format) and with standard and affordable read-out equipment (photo scanner) amending some limitations of other miniaturized platforms.

2. Results and Discussion

2.1. Screening Workflow on DMA

DMA slides patterned with an array of hydrophilic spots separated by superhydrophobic borders have been used in this research project. The DMA slides had a 7.5 × 2.5 cm dimension containing 588 squares hydrophilic spots with 1 mm side length. Due to the precise dimension of spots and stable superhydrophobic borders, homogeneous bacterial droplet arrays could be generated by printing bacteria suspensions directly into each spot using a non-contact liquid dispenser. As shown in **Figure 1**a, solutions of compounds in DMSO were first printed onto DMA slides with a liquid dispenser. Afterwards slides were dried in a desiccator overnight. Then, 150 nL bacteria suspension of *K. pneumoniae* or MRSA was printed onto each hydrophilic spot to form droplets. Bacteria were incubated with the compounds in droplets overnight. Then droplets were stained with Cell Counting Kit-8, which is transparent water-soluble tetrazolium salt 8 (WST-8) and reduced to bright yellow to orange WST-8 formazan by living bacteria. Color change is proportional to concentration of living bacteria in the droplets and inhibition of the growth of bacteria in droplets could be detected visibly. For the analysis of color change in the droplets, we have acquired a digital image of the whole array using a conventional photo scanner. The whole DMA slide containing 588 experiments can be scanned in six minutes and color depth can be analyzed in obtained digital image by using in house developed automated protocol.

2.2. Printing and Culturing of *K. pneumoniae* and MRSA on DMA

To investigate the influence of the printing process on the viability of bacteria in suspension, a LIVE/DEAD assay was applied to detect living or dead *K. pneumoniae* after the printing. Living bacteria were identified with a green fluorescence after SYTO9 staining, and dead bacteria – with red fluorescence after propidium iodide (PI) staining with the help of epifluorescence microscopy. As shown in Figure 1b, the viability of bacteria immediately after printing measured from the LIVE/DEAD

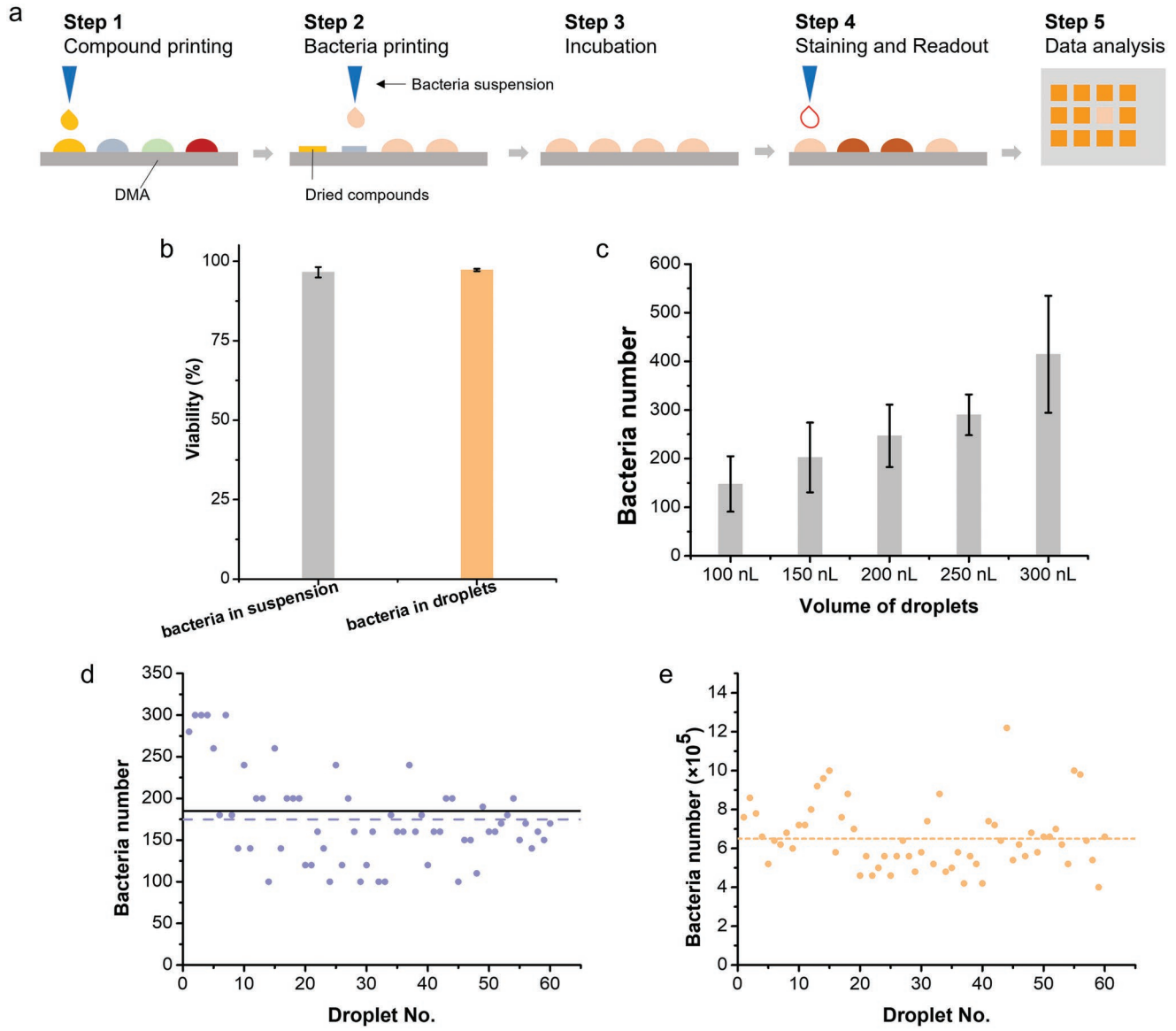


Figure 1. DMA as a screening platform to culture drug-resistant bacteria in droplets. a) Schematic illustration of the workflow of HTS of compounds with *K. pneumoniae* and MRSA using DMA: compounds printing → drying → bacteria printing→ incubation → printing of staining solutions → colorimetric readout by a scanner → data analysis. b) Viability of *K. pneumoniae* before and after the printing. $n = 10$. Data were presented as mean ± SD of three experiments. c) Number of *K. pneumoniae* in droplets of different volumes. $n = 3$. Data were presented as mean ± SD of three experiments. d) Number of bacteria in 150 nL droplets on DMA, estimated by colony counting experiments. The content of 60 droplets was analyzed. The horizontal black solid line shows the desired number of bacteria in single droplets estimated according to initial bacteria density and printing volume. The blue dash line shows the actual number of bacteria in single droplets obtained from experimental data. e) The number of bacteria per droplets after 18 h incubation estimated by colony counting experiments. The content of 60 droplets was analyzed. The yellow dash line shows the average number of bacteria in single droplets obtained from experimental data (b) and (c).

assay was 97.2 ± 0.3%, close to the viability of bacteria in initial bacteria suspension, which was 96.5 ± 1.6%. The result indicates that the printing process caused no obvious destruction to bacteria. To investigate if an actual number of bacteria in single droplets correspond to desired one based on density of bacteria suspension before printing, 60 droplets containing bacteria from three DMA slides were collected and a colony counting method was used to estimate the bacteria number. As shown in Figure 1c, the average number of bacteria in each droplet was 174.8 ± 57.8, whereas desired number of bacteria per droplets was 185.0 ± 8.6, which was calculated according to the bacteria density of initial suspension and printing volume. Figure 1d shows that bacteria proliferated overnight in droplets and reached a high bacteria density of $6.5 \pm 1.7 \times 10^5$ bacteria per droplet, confirming that *K. pneumoniae* can proliferate in droplets on DMA. For MRSA, the average number of bacteria in printed droplets was 282.5 ± 87.8 (compared to calculated 270.0 ± 41.0 per droplet); and the density of MRSA after overnight incubation increased to $4.8 \pm 2.0 \times 10^4$ bacteria per droplet (Figure S2b, Supporting Information). Taking together our results demonstrate that bacteria viability is not influenced by printing (Figure 1b); that we can obtain homogeneous

distribution of number of bacteria per droplets, and control this number by manipulating initial concentration of bacteria and printed volume (Figure 1e), and that bacteria proliferated in 150 nL droplets in overnight incubation (Figure 1d).

2.3. Colorimetric Readout on DMA using Cell Counting Kit-8

A colorimetric readout method has been developed to analyze the screening results and be able to detect bacteria growth inhibition in droplets in a convenient, rapid, and cost-saving manner. Cell Counting Kit-8 based on reduction of WST-8 by living bacteria is widely used in proliferation and cytotoxicity assays, where proportion of living bacteria can be estimated by measuring optical density of reduced WST-8.[14] Droplets of 150 nL containing different bacteria numbers were generated on DMA. Then a staining solution of 100 nL was added into droplets and incubated for 1 h. The stained DMA slide was then scanned by a flatbed document scanner. Within six minutes, a scan image of all droplets was achieved for further data analysis. No expensive equipment or professional skills are required for this readout step, allowing a rapid and cost-efficient data collection for the high throughput screening with bacteria. **Figure 2**a,b shows the scan images and corresponding color depth values of stained droplets. With the decreasing of bacteria number in droplets a color change

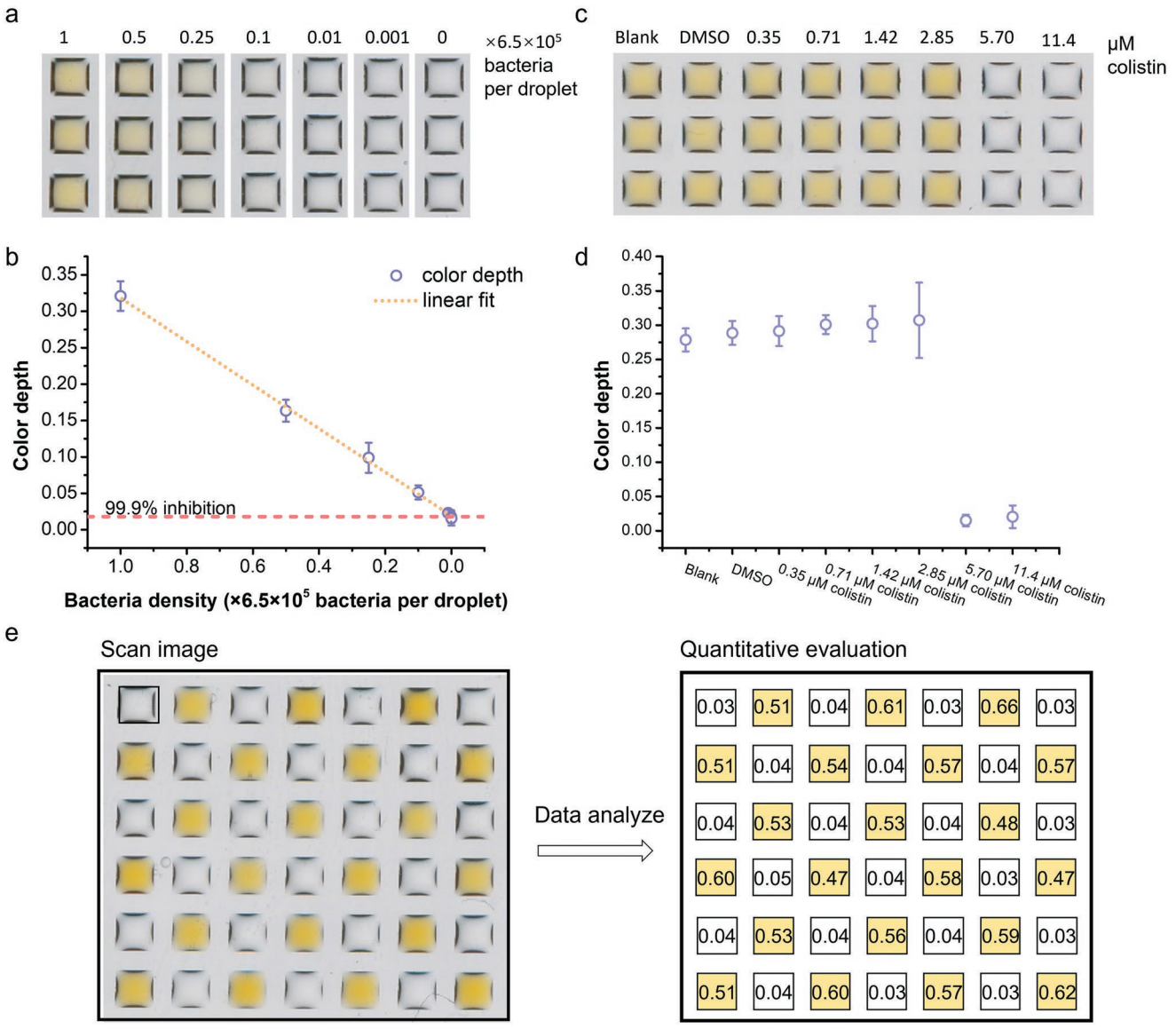


Figure 2. Colorimetric readout method to evaluate the growth of *K. pneumoniae* in droplets using Cell Counting Kit-8. a) Scanned image of droplet arrays containing different numbers of bacteria stained with Cell Counting Kit-8 solution. b) The color depth of stained droplets shown in (a). $n = 16$. Data were presented as mean ± SD of three experiments. c) Scanned image of droplet arrays with empty control, DMSO and colistin in different concentrations, containing *K. pneumoniae* cultured on the array overnight and stained with Kit-8. d) The color depth of stained droplets shown in (c). $n = 10$. Data were presented as mean ± SD of three experiments. e) Scanned image of droplet arrays with DMSO and colistin printed in checker board pattern containing *K. pneumoniae* cultured on the array overnight and stained with Kit-8 (left panel). Color depth of droplets shown in the scanned image (right).

from bright orange to almost transparent was observed. The droplets containing bacteria of low density (650 bacteria per droplet), which was close to the density of initial bacteria suspension, can be easily distinguished from the droplets containing bacteria of high density, which was the same density of overnight cultured droplets ($6.5 \pm 1.7 \times 10^5$ bacteria per droplet) (Figure 2a,b). The lowest bacteria density that could be detected with this method was $0.65 \pm 1.7 \times 10^5$ bacteria per droplet, which is 0.1% of high density bacteria without treatment. Therefore, with our method we can detect up to 99.9% of growth inhibition rate making it suitable for screening for potential antimicrobial compounds. Figure S2c,d in the Supporting Information show that this colorimetric readout method can also be applied to MRSA.

2.4. Validation of the Workflow for High Throughput Screening on DMA

As a first step, we have identified negative (solvent) and positive controls for high throughput screening of both *K. pneumoniae* and MRSA on DMA. Figure 2c,d and Figure S2c (Supporting Information) show that 1.5 nL of 100% DMSO (1% of final culturing volume) used as a solvent for compounds, did not influence the growth of *K. pneumoniae* and MRSA in comparison with empty control. This indicates the use of DMSO as a solvent for compounds not causing any false-positive results. DMSO was then applied as a negative solvent control in the following screening experiments. As a positive control for *K. pneumoniae* antibiotic colistin sulfate was used in 5.7×10^{-6} M concentration, which showed more than 99.9% of bacteria growth inhibition compared with DMSO control (Figure 2c,d). The color depth of positive control droplets was 0.01 ± 0.02, whereas the color depth of empty and DMSO control was 0.27 ± 0.02 and 0.28 ± 0.02, respectively (Figure 2c,d). Figure 2e shows that there was no contamination between droplets incubated with and without colistin. As a positive control for MRSA antibiotic fosfomycin in concentration of 54.3×10^{-6} M was used, showing 99.9% of bacteria growth inhibition (Figure S2e, Supporting Information). According to the determination of the *Z*-factor value, which is used to estimate the performance of screening assays, the *Z* factor of the colorimetric assay on DMA was 0.73, indicating that it is an excellent assay.[15]

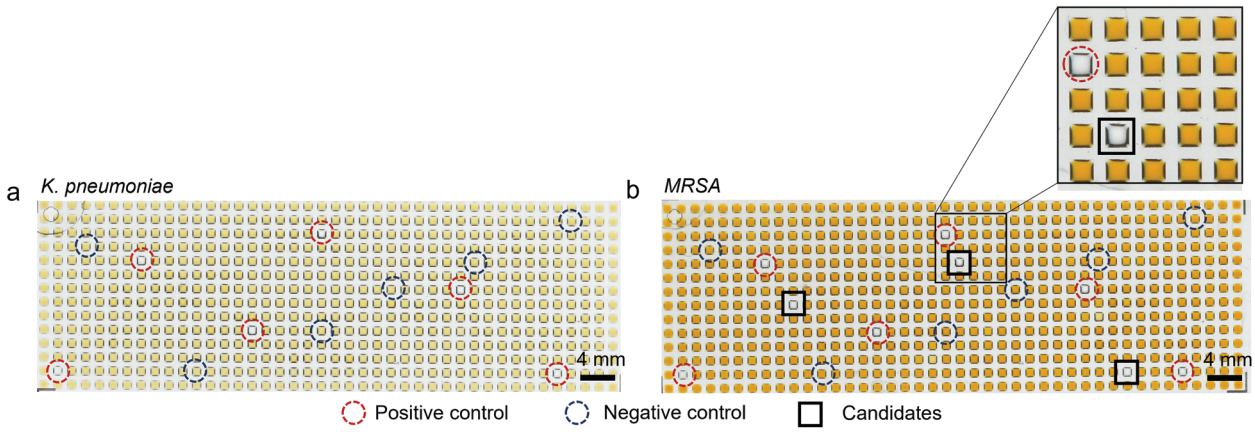
2.5. High Throughput Screening of 2826 Compounds with *K. pneumoniae* and 2060 Compounds with MRSA

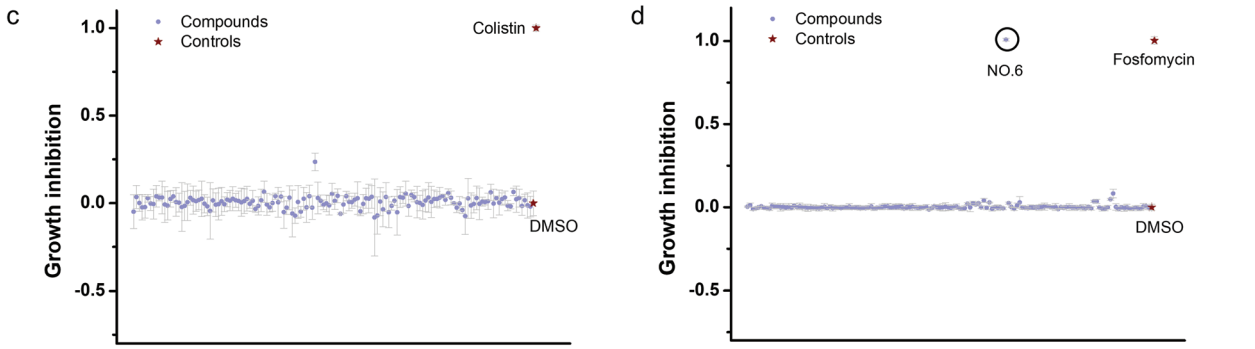
Using the established screening assay, the screening of compound libraries containing 2826 and 2060 unique new compounds were screened for their ability to inhibit the growth rate of *K. pneumoniae* and MRSA, respectively. The majority of the tested compounds have drug-like properties according to Lipinski's rule of five (RO5).[16] The molecular weight of 82.83% of the compounds is below 500 Da, whereas the octanol-water partition coefficient (log *P*) of most compounds is in the range of −0.4 to +5.6 (extended RO5) (79.51% of the compounds). Meanwhile, the topological polar surface area (TPSA) of 95.25% out of the total compounds is below 140 Å^2, while in turn, 83.11% of all compounds have a TPSA of less than 90 Å^2. (Figure S1, Supporting Information) The libraries of 2826 and 2060 compounds were printed on 20 and 15 DMA slides, respectively. Each DMA slide contained six positive controls, six negative controls, nine blank controls, and three repetitions of each compound, all of which were randomly distributed across DMA slide to avoid the bias of a position. The scans of one DMA with 152 compounds containing cultured and stained *K. pneumoniae* and MRSA are shown in **Figure 3**a,b, respectively. As expected, the droplets containing positive controls were transparent, while negative controls showed orange color, demonstrating the validity of the screening process using DMA. In Figure 3a, all droplets except positive controls presented orange color, implying that no hit compounds have been found on this DMA slide among tested 152 compounds. While in Figure 3b, three transparent droplets were detected. The value of color depth of droplets on the slide was analyzed by a program developed using MATLAB and then normalized against the controls. The inhibition rate was obtained to evaluate the antibacterial effect of compounds. Figure 3c shows the inhibition rate of compounds printed on the DMA slide in Figure 3a, demonstrating that none from tested compounds showed activity against *K. pneumoniae*. Meanwhile, Figure 3d shows that compound NO.6 inhibited the growth of MRSA after overnight incubation, which is consistent with the results shown in the scanned image of Figure 3b.

Results of screening of full libraries are demonstrated in Figures S3–S5 in the Supporting Information. According to the results of the screening, six compounds were identified as positive hits against MRSA (inhibition rate > 0.9). The structure and inhibition rate of all identified candidates against MRSA are presented in Figure 3e. However, no candidates with the testing concentration 10×10^{-6} M against *K. pneumoniae* with inhibition rates comparable to positive controls were identified. *K. pneumoniae* is a Gram-negative bacteria and intrinsically resistant to existing antibiotics. A common mechanism involved in intrinsic resistance is a reduced permeability of the outer membrane of Gram-negative bacteria, which limits the uptake of certain molecules into the cell.[17] The *K. pneumoniae* strain ATCC BAA-2146 used in this study was reported to possess genes associated with resistance to β-lactams, fluoroquinolones, aminoglycosides and folate pathway inhibitors.[18] In addition, it was shown that *K. pneumoniae* contains efflux pumps from different families to remove toxic substances from inside the cells.[19] The broad spectrum of resistance mechanisms of *K. pneumoniae* makes it difficult to identify new compounds able to inhibit the growth of this bacterium. More compounds designed for drug-resistant bacteria, or compounds with higher concentrations could be screened for candidates active against *K. pneumoniae*.

2.6. Minimum Inhibitory Concentration (MIC) Assay to Validate Screened Candidates

All compound candidates from the screening were further validated in microtiter plates by determining the MIC of the compounds. Each compound was tested in five concentrations ranging from 1.25 to 20×10^{-6} M. As shown in **Figure 4**, all





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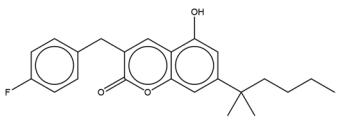
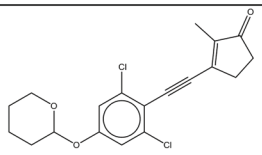
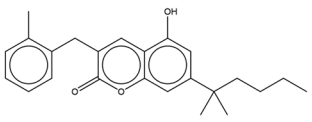
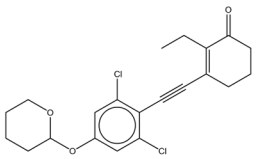
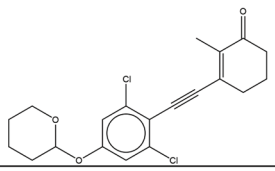
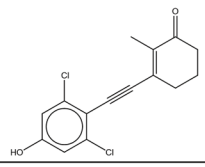
Compound NO.	Structure	Inhibition rate	Compound NO.	Structure	Inhibition rate
1		0.98±0.01	4		1.00±0.02
2		0.97±0.01	5		0.99±0.01
3		1.01±0.01	6		1.00±0.01

Figure 3. Preliminary screening of compounds on DMA. a) Scanned image of stained droplets containing *K. pneumoniae* incubated overnight on DMA printed with 152 compounds. Positive control: colistin, 5.7×10^{-6} M. Negative control: DMSO. The concentration of compounds in the screening: 10×10^{-6} M. b) Scanned image of stained droplets containing MRSA incubated overnight on DMA printed with 152 compounds. Positive control: fosfomycin, 54.3×10^{-6} M. Negative control: DMSO. The concentration of compounds in the screening: 10×10^{-6} M. In (a) and (b), droplets of positive control are marked with red dotted circles. Droplets of negative control are marked with blue dotted circles. Compounds inhibiting the growth of bacteria are marked with black solid squares. c) The inhibition rate of compounds tested on the DMA slide shown in (a). $n \geq 3$. Data are presented as mean ± SD. d) Inhibition rate of compounds tested on the DMA slide shown in (b). $n \geq 3$. Data is presented as mean ± SD. Inhibition rate is obtained by normalizing the color depth of droplets against the color depth of droplets of positive controls and negative controls. e) Structures and inhibition rates of hit compounds identified in the screening.

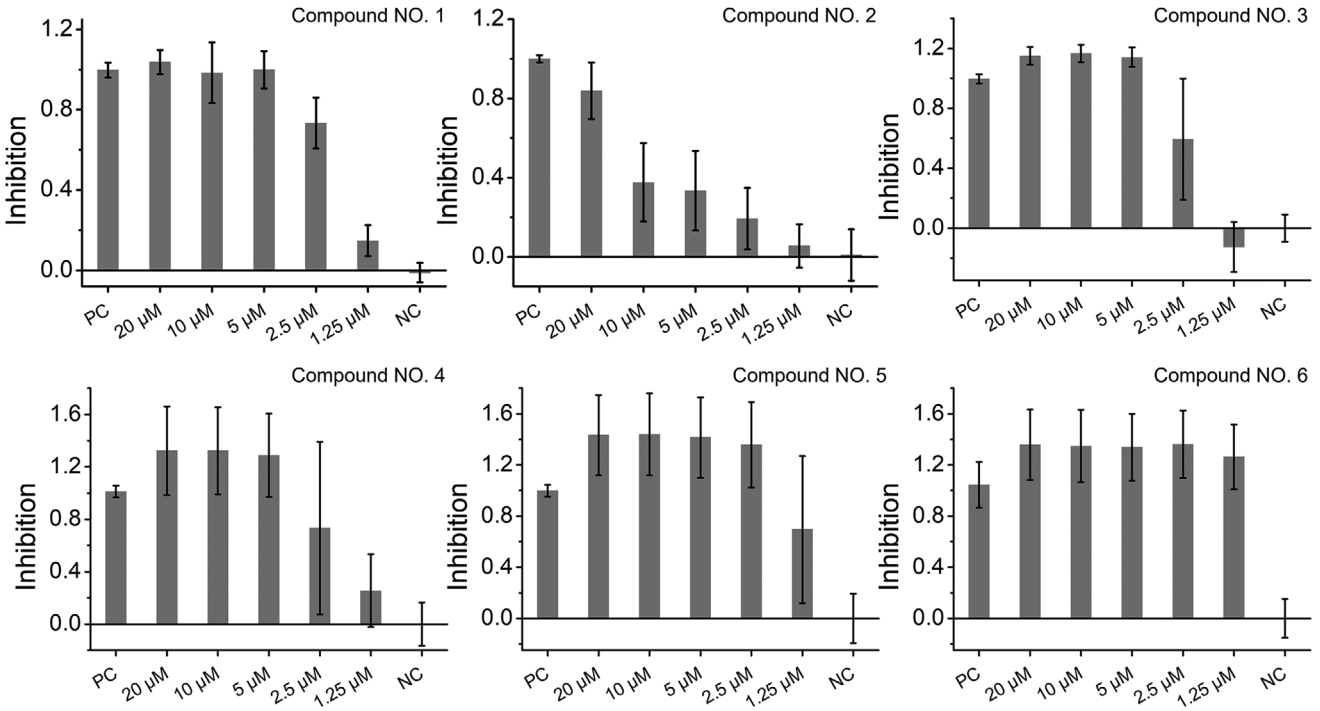


Figure 4. Determination of MICs of six compound candidates in microplates using MRSA. Positive control (PC): fosfomycin, 72.4×10^{-6} M. Negative control (NC): DMSO. $n = 5$. Data were presented as mean ± SD of three experiments.

six compound candidates against MRSA were successfully validated using MIC test. The MIC of compound No.1 to compound No.6 are 5, 20, 5, 5, 2.5, and $\leq 1.25 \times 10^{-6}$ M.

Two classes of molecules stood out as potential antimicrobials against MRSA. The first compound class has a coumarin core structure (compounds No.1 and No.2). Coumarins are known to have different biological activities, such as anticoagulant, antioxidant, antimicrobial, anticancer, and antidiabetic to name only a few.[20] Studies have reported that coumarins could kill bacteria by damaging the cell membrane.[21] However, extensive structure–activity relationships (SAR) need to be established to make reliable statements about the influence of functional groups on the bactericidal activity of these compounds. The hits (compounds No.3–No.6) from the second compound class of interest are structurally simplified estrogen analogs.[22] Estrogen is a hormone that plays various roles in the body and presents a wide range of biological functions. However, antibacterial activity has not been reported yet for estrogen or simplified estrogen analogs. The mechanisms of action of MSRA inhibition by compounds No.3–No.6 need to be further studied and elucidated. Thus, new structures of antibacterial molecules have been identified by the screening using the DMA-based system, suggesting that it is a promising miniaturized platform for the screening of antibacterial compounds.

3. Conclusion

In this study, the workflow of HTS of antimicrobial compounds using DMA has been established and validated. It was demonstrated that low volume liquid dispensers can be applied to generate droplets containing a defined number of bacteria. Droplets containing hit compounds, which lead to inhibition of bacterial growth, could be easily distinguished visibly from droplets containing a high density of bacteria. A simple colorimetric readout method was developed and validated. With a simple paper scanner, a DMA slide with 588 droplets (152 tested compounds) can be screened in a few minutes to provide quantitative data of the color depth of stained droplets.

The HTS workflow developed in this research opens opportunities to identify antimicrobial agents from other libraries to provide new therapies to treat drug-resistant infections. In total, 2826 compounds from the DFG core facility Molecule Archive of the Compound Platform have been screened against *K. pneumoniae* and 2060 compounds have been screened against MRSA using this HTS system. Six candidates were identified and validated by a MIC assay in microtiter plates, providing novel compounds against multidrug-resistant bacteria.

4. Experimental Section

Materials and Instruments: Patterned superhydrophobic-hydrophilic glass slides (7.5 × 2.5 cm) were obtained from Aquarray GmbH (Eggenstein–Leopoldshafen, Germany). Ethanol was from Merck (Darmstadt, Germany). Müller–Hinton (MH) medium was purchased from Merck (Darmstadt, Germany). Colistin sulfate and fosfomycin were purchased from Sigma-Aldrich (Munich, Germany). Cell Counting Kit-8 was purchased from MedChemExpress (New Jersey, USA).

The AxioImage M2 system equipped with an Apotome (Carl Zeiss, Oberkochen, Germany) was used for fluorescence microscopy.

CanoScan 8800F was used to scan DMA slides. Noncontact liquid dispenser SciFlexarrayer S11 (Scienion AG, Germany) and I-DOT (CELLINK, Stuttgart, Germany) were used to print compounds on DMA. Noncontact liquid dispenser I.DOT MINI (CELLINK, Stuttgart, Germany) was used to print bacteria suspension.

Printing of Compounds on DMA Slides: All compounds were obtained as 10×10^{-3} M stock solution in DMSO from the library of the Molecule Archive. The compound solution (1.5 nL) was printed onto individual hydrophilic spots by SciFlexarrayer S11. DMA printed with compounds were placed in a desiccator overnight at room temperature (≈25 °C). Then the slides were removed from the desiccator for bacteria printing. Colistin sulfate and fosfomycin were dissolved in water to a concentration of 1 mg mL^{-1} and then diluted for further printing. The DMA printed with antibiotics were processed in the same way as the DMA printed with testing compounds.

Live/Dead Staining of Bacteria: *Klebsiella pneumoniae* ATCC BAA-2146 colonies were picked up from LB agar plates and inoculated into MH medium for overnight culture. Bacteria suspension (150 nL) of the overnight culture was printed on individual hydrophilic spots on DMA slides. Then 10 droplets were collected by pipetting to MH medium (0.1 mL) and stained with SYTO9 (0.15 µL) and PI solution (0.075 µL) from the LIVE/DEAD BacLight Bacterial Viability Kit. After 15 min incubation in the dark at room temperature, stained bacteria suspension was moved to microscope slides and observed with epifluorescence microscope. Live/dead staining of initial bacteria suspension was processed in the same way as printed bacteria suspension.

Colony Counting Tests of Bacteria: Colonies of *Klebsiella pneumoniae* and MRSA were picked up from LB agar plates and inoculated into MH medium for overnight culture. The optical density of cultured bacteria suspension was measured. Bacteria suspension was diluted with MH medium to the calculated OD_{600} value = 0.001, corresponding to 1.2×10^6 CFU mL^{-1} for *Klebsiella pneumoniae* and OD_{600} value = 0.01, corresponding to 1.8×10^6 CFU mL^{-1} for MRSA. *Klebsiella pneumoniae* and MRSA bacterial suspension (150 nL) was printed on individual spots on DMA. Single droplets of bacteria suspension on DMA were then removed by pipetting to 1 mL cell wash buffer and diluted 1:100 with PBS solution. Afterward, prepared suspension (10 µL) was inoculated to MH agar plates and incubated statically at 37 °C for 18 h. The colony number of inoculated bacteria was counted, which was used to calculate the bacteria density in droplets.

Printing of Bacteria on DMA Slides: Colonies of *Klebsiella pneumoniae* and MRSA were picked up from LB agar plates and inoculated into MH medium for overnight culture. The optical density of cultured bacteria suspension was measured. Bacteria suspension was diluted with MH medium to the calculated OD_{600} value = 0.001 for *Klebsiella pneumoniae* and OD_{600} value = 0.01 for MRSA. Prepared bacteria suspension (150 nL) was printed onto individual spots on DMA using a liquid dispenser of type I.DOT MINI. Then DMA slides with compounds and bacteria were incubated statically at 37 °C for 18 h. To prevent evaporation, DMA slides were placed in a sealed box, with a piece of wet tissue inside to create high humidity.

Colorimetric Readout Method using Kit8: After incubation of DMA slides, Cell Counting Kit-8 solution (100 nL) was printed to individual droplets on DMA slides with I.DOT MINI. Then slides were incubated for another 1 h in the humidity box. Afterward, DMA slides were placed into a paper scanner to scan the whole slide using the positive-film scan function. High-resolution images (6400 dpi) were generated for the next data analysis.

The color depth of each droplet was analyzed with MATLAB R2020b using a program provided by Prof. Markus Reischl. For analysis of scanned images of DMA slides stained with WST-8, the user defined the number of rows and columns of droplet spots to be evaluated, as well as the top left corner, bottom right corner, horizontal and vertical displacement of the spot arrays. The central position of all droplet spots was estimated and a square of 41×41 pixels located close to the center was extracted. The intensity of the blue color-channel was calculated, inverted and normalized to [0,1]. The intensities were then averaged within the extracted square to obtain a single intensity value.[13]

MIC Assay: Colonies of MRSA were picked up from LB agar plates and inoculated into MH medium for overnight culture. The optical density of cultured bacteria suspension was measured. Bacteria suspension was diluted with MH medium to the calculated OD_{600} value = 0.01. Bacteria suspension was added in individual wells of 96-well plates by pipetting. Compounds and fosfomycin were added to each well by pipetting to prepare solutions of certain concentrations (as shown in Figure 4). Plates were incubated in a humidity box overnight statically at 37 °C. The OD values of samples were measured and normalized by positive controls and negative controls, then presented as inhibition rate

$$\text{Inhibition} = \frac{OD_{test} - OD_{negative}}{OD_{positive} - OD_{negative}} \quad (1)$$

Statistical Analysis: All data were represented as mean ± SD of $n \geq 3$ individual repetitions for each experiment. Experiments were at least repeated three times. The final screening of compound libraries containing 2826 and 2060 compounds using *K. pneumoniae* and MRSA was conducted once. The fitting curve in Figure 2b was plotted in OriginPro using "linear curve fit" function. The color depth of each droplet was analyzed with MATLAB R2020b using a program provided by Prof. Markus Reischl. The normalization in Figures 3c–e and 4 was obtained by

$$value_{normalized} = \frac{value_{test} - value_{negative}}{value_{positive} - value_{negative}} \quad (2)$$

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in the Chemotion repository and RADAR4KIT at https://radar.kit.edu/radar/en/dataset/lNXUHmtzbOWhoBET, https://doi.org/10.14272/collection/SGV_2022-05-12, and https://doi.org/10.14272/collection/SGV_2022-05-03.[23–25]

Keywords

antibacterial, high throughput screening, *Klebsiella pneumoniae*, microarrays, MRSA

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