

Nanomolar Synthesis in Droplet Microarrays with UV-Triggered On-Chip Cell Screening

Marius Brehm, Stefan Heissler, Sergii Afonin, and Pavel A. Levkin*

Miniaturization and parallelization of combinatorial organic synthesis is important to accelerate the process of drug discovery while reducing the consumption of reagents and solvents. This work presents a miniaturized platform for on-chip solid-phase combinatorial library synthesis with UV-triggered on-chip cell screening. The platform is based on a nanoporous polymer coating on a glass slide, which is modified via photolithography to yield arrays of hydrophilic (HL) spots surrounded by superhydrophobic (SH) surface. The combination of HL spots and SH background enables confinement of nanoliter droplets, functioning as miniaturized reactors for the solid-phase synthesis. The polymer serves as support for nanomolar solid-phase synthesis, while a photocleavable linker enables the release of the synthesized compounds into the droplets containing live cells. A 588 compound library of bisamides is synthesized via a four-component Ugi reaction on the chip and products are detected via stamping of the droplet array onto a conductive substrate and subsequent matrix-assisted laser desorption ionization mass spectrometry. The light-induced cleavage shows high flexibility in screening conditions by spatial, temporal, and quantitative control.

1. Introduction

Discovering new drugs is a major challenge and can take more than 20 years of work producing costs of over 2 billion dollars per drug.^[1] These expenses do not include the costs of the synthesis of compound libraries, which are critical for the drug discovery process. In order to solve this important problem, the process of the synthesis of compound libraries and their biological screenings has to be drastically changed. The new process should be compatible with a) highly parallel synthesis; b) miniaturization, synthesis in submicroliter volumes; and c) the same platform should be compatible with both library synthesis and biological screenings. Such a platform may significantly reduce the amounts of used chemicals, solvents, and biological material and accelerate the whole process via parallelization of synthesis and integration of chemistry and biology. For example, the simple switch

from a 96-well plate to a 384-well format decreases the sample volume by a third and increases experimental density by four.

Various platforms addressing miniaturized high-throughput synthesis and screenings have been developed. Frank introduced the SPOT technique, where a cellulose membrane served as the solid support for a stepwise, combinatorial, and parallel peptide synthesis.^[2] Over the years, the scope has also been expanded to other chemistries like small molecules.^[3] The low price of the cellulose and easy handling are important advantages of this platform. The size of the spots, however, is determined by the pipetted volume of the reaction solution, which leads to the risk of cross-contamination or incomplete coupling if too much or too little solution is applied.^[4] The use of the synthesized compounds in a solution-based assay after cleavage from the support is also laborious and difficult to miniaturize.^[5] With standard liquid handling robotics, an array of peptides with a density of up to 14 peptides per cm² could be achieved.^[6,7] To overcome the problems of liquid handling, Breitling et al. developed a solvent-free laser printer-based peptide array synthesis,^[8,9] increasing peptide densities to 40 000 cm⁻² and applying them to study protein–antibody interactions.^[8] The limitation of this method is that produced peptides stay bound to the surface making solution-based biochemical or cell-based assays difficult without cross-contamination.

The droplet microarray (DMA) is a platform for miniaturized high-throughput cell screening in volumes down to several nanoliters, which has been exploited in diverse biological applications.^[10,11]

M. Brehm, Dr. P. A. Levkin
Karlsruhe Institute of Technology (KIT)
Institute of Toxicology and Genetics
Hermann-von Helmholtz-Platz 1
76344 Eggenstein-Leopoldshafen, Germany
E-mail: pavel.levkin@kit.edu

S. Heissler
Karlsruhe Institute of Technology (KIT)
Institute of Functional Interfaces
Hermann-von Helmholtz-Platz 1
76344 Eggenstein-Leopoldshafen, Germany

Dr. S. Afonin
Karlsruhe Institute of Technology (KIT)
Institute of Biological Interfaces (IBG-2)
POB 3640, 76021 Karlsruhe, Germany

Dr. P. A. Levkin
Karlsruhe Institute of Technology (KIT)
Institute of Organic Chemistry
Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/smll.201905971>.

© 2020 The Authors. Published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

DOI: 10.1002/smll.201905971

It is usually based on a support substrate (e.g., glass) coated with a thin nanoporous polymer layer, which is spatially modified via UV-induced thiol-yne reactions to yield hydrophilic (HL) spots on a superhydrophobic (SH) background. The HL spots can confine liquid droplets with volumes from nanoliters to microliters depending on the size. We recently reported the use of the DMA platform for liquid-phase chemistry, where the droplets served as microvessels to synthesize a library of lipidoids, which were screened for transfection efficiency.^[12] Introducing a photocleavable linker to couple the starting material to the polymer layer of the DMA allowed us to synthesize tripeptides via solid-phase peptide synthesis as the first multistep synthesis performed on this platform.^[13] However, the attachment of the compound to the solid support of the HL spot has more advantages such as simple purification by washing off the reaction solution or the possibility to immerse the whole DMA in reaction mixtures or sterilization solutions without cross-contamination.

With this work, we expand the scope of the platform to more complex chemistry such as the Ugi four-component reaction^[14] and further investigate the possibility of on-chip cellular screenings. The Ugi reaction is one of the most famous multi-component reactions and is widely used for rapid library creation. Multicomponent reactions, in general, can yield the largest number of different products for the smallest input of variation.^[15] For example, a two-compound reaction with 20 educts can give 400 different products (20^2), while a four-component reaction with 20 different building blocks can give up to 160 000 (20^4). The core structure in the case of the Ugi reaction is a bisamide, and the substituents of it are variable. When tested for affinity to a biological target, more different substituents may give more positive hits for further improvement and information about the structure–activity relationship, so more extensive libraries are of an advantage. Parallelization and miniaturization are the bottlenecks of this research, which inspired us to improve high-throughput chemical library synthesis with the present work. We used the droplet microarray platform to perform a combinatorial solid-phase synthesis of a nanomolar library of over 500 different compounds, followed by photorelease from the surface and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) analysis. Biological experiments could be performed directly after the synthesis on the same chip. Due to the transparency of the surface, microscopic readouts are possible. We show that initially surface-bound compounds are only affecting the cells after the UV-triggered release, thus adding high flexibility to the screening conditions in terms of spatial, temporal, and quantitative control.

2. Results and Discussion

2.1. Surface Characteristics

The DMA consists of a microscopic glass slide in the size of $76 \times 26 \times 1$ mm, which is covered with a layer of a nanoporous polymer (poly(2-hydroxyethyl methacrylate-co-ethylene dimethacrylate, HEMA-co-EDMA). Topmost nonporous part of the polymer is removed by the adhesive tape to improve the hydrophobicity and permeability. The thickness of the coating was controlled by the amount of polymerization mixture (35 μ L)

and yielded around 12 μ m. The HEMA-co-EDMA polymer surface was patterned via sequential esterification with 4-pentynoic acid, followed by the UV-induced thiol-yne click reaction with either cysteamine hydrochloride or 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiol to form HL round ($d = 3$ mm) or square ($b = 1$ mm) spots surrounded by SH borders (Figure 1A). The static water contact angle of the HL spots was measured as $\theta_{\text{st}} = 4.4^\circ$. For the SH background, values were $\theta_{\text{st}} = 170^\circ$, $\theta_{\text{adv.}} = 173^\circ$, and $\theta_{\text{rec.}} = 164^\circ$.^[16] This big difference in wettability allows for the effect of discontinuous dewetting of aqueous solutions, leading to an array of strictly separated droplets with the volume of around 3–5 μ L (3 mm, round) or 80–100 nL (1 mm, square).^[17,18] The volume held in the spots during discontinuous dewetting depends on the surface tension of the solvent and is generally lower for organic solvents. Some organic solvents, such as dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), γ -butyrolactone (GBL), and dimethylsulfoxide (DMSO), although being lower surface tension liquids compared to water, form stable droplets on the polymer surface when they are pipetted into the HL spots. However, since their surface tension is lower, the formed droplets are smaller, and the discontinuous dewetting effect can be disturbed by minute surface damages or inhomogeneity of its chemistry, which in turn leads to merging of droplets over the SH border.

Hydroxyethyl photolinker ([4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy]-butanoic acid, HEPL) was chosen as the anchor to the solid phase since it can be rapidly cleaved under aqueous conditions.^[19] It is reactive to UV light of 365 nm, which has been shown in the literature to be nontoxic for cells.^[20] As shown in Figure 2A, carboxylic acids can be coupled to its benzylic hydroxyl group via standard esterification protocols and released again when required via UV irradiation as the free carboxylic acid, leaving the cleaved linker (c-HEPL) on the solid phase.

2.2. Photorelease

We investigated the kinetics of the cleavage by calculating the amount of chlorambucil (CHL, Figure 2A) released into the droplets after different irradiation times. CHL was loaded onto the linker according to the procedure described in the Experimental Section and each four spots were irradiated with UV light at 365 nm for a certain time. The combined cleavage solution of the four spots was diluted and measured for its absorbance at 284 nm, which refers to the wavelength of the previously determined extinction coefficient ($\epsilon = 1950$ L mol⁻¹ cm⁻¹, Figure S3, Supporting Information). The loading was determined using the Beer–Lambert law as nmol mm⁻² and plotted against irradiation time in Figure 2B. The linker's half-life (irradiation time when half of the molecules are released) under UV exposure at 365 nm with 2.5 mW cm⁻² was measured to be around 7.5 min. The rapid photolytic cleavage of the linker in an aqueous environment is important for the application of UV-induced drug release process under biologically relevant conditions. This half-life is comparable with values for UV release in the liquid phase known from the literature.^[19] We achieved a maximum loading of CHL of 0.68 nmol mm⁻² in a 3 mm round spot, which was completely released into the droplets after 15 min of UV irradiation (365 nm, 2.5 mW cm⁻²). The area of a circle with a diameter

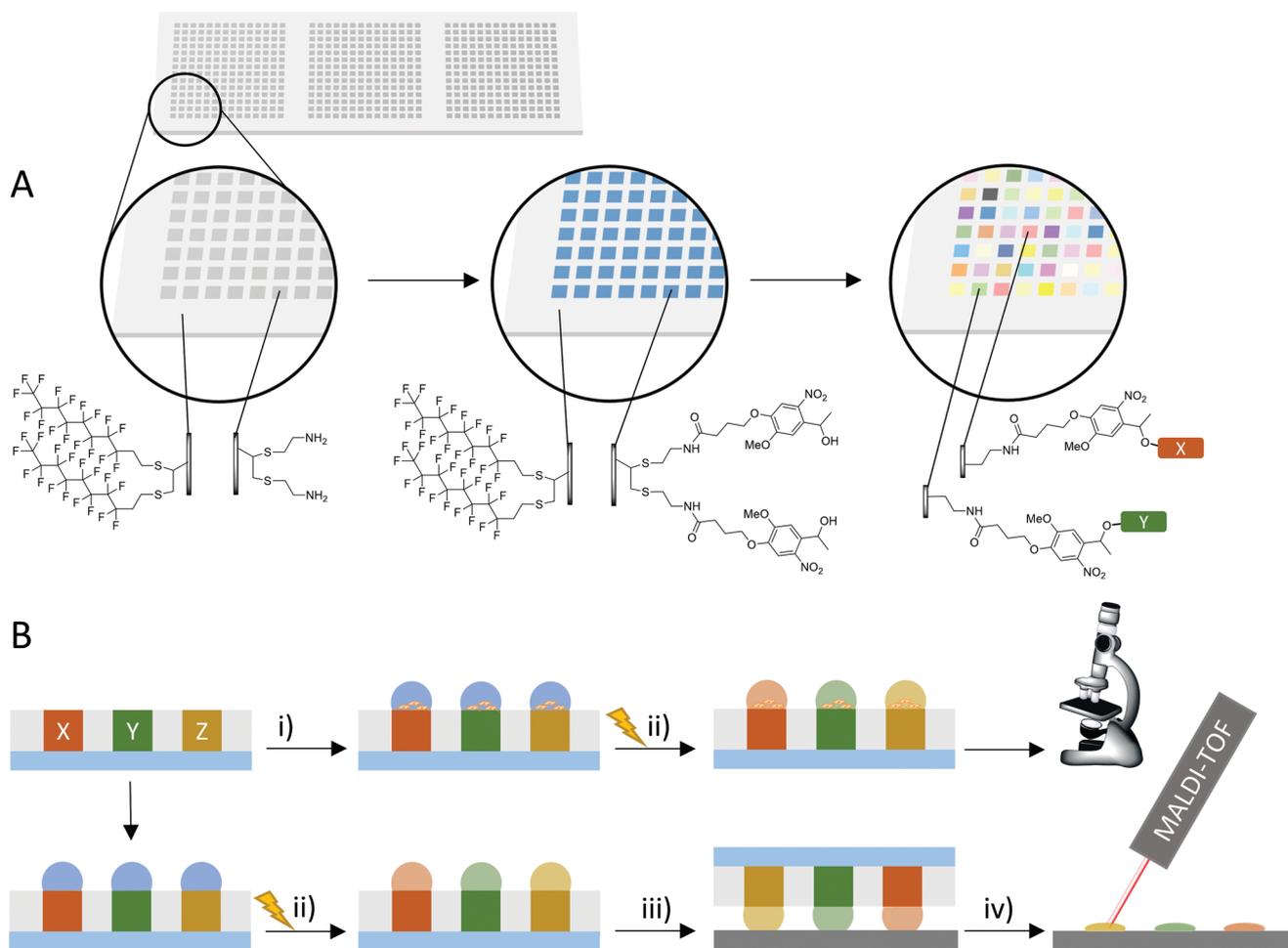


Figure 1. Droplet microarray platform combining high-throughput solid-phase synthesis and cell-based screenings. A) Porous polymethacrylate thin film (12 μm) is functionalized with fluoroalkyl and cysteamine moieties to generate SH and reactive HL regions, respectively. UV-cleavable linker (hydroxyethyl photolinker, HEPL) is covalently immobilized in the HL spots, forming the basis for the following solid-phase combinatorial synthesis. B) Scheme showing possible cell-based screening sequence: synthesized compounds can be released by UV irradiation at 365 nm ii) into distinct nanodroplets formed in the HL spots to be either screened in biological assays (i) or analyzed, e.g., via MALDI-TOF mass spectrometry (iv) following a sandwiching step (iii).

of 3 mm (7.01 mm²) can hold 4.77 nmol of CHL. When the spot is filled with 5 μL of solvent, this leads to a maximum accessible concentration of 954×10^{-6} M. According to the literature, the recommended concentration for high-throughput hit discovery is $1\text{--}10 \times 10^{-6}$ M,^[21–23] showing that the amount of compound in each spot is enough for biological screenings even in cases of yields less than 100%. The nanoporous HEMA-co-EDMA polymer is opaque when it is dry, but turns transparent when wetted in the HL spots due to the reduction of light scattering. Because of this transparency and the thin coating, the UV light can access the whole reactive areas on the polymer without getting blocked. When compared to nontransparent cellulose-based platforms like SPOT and their ammonia- or phototriggered release, our photorelease is 20 times or six times faster, respectively.^[24,25] Besides, dry cleavage is essential in the case of other arrayed synthetic platforms to keep the format intact, since there are usually no self-separating and predefined droplet patterns like on the DMA.

Light can easily be controlled by focused beams or photo-masks to spatially irradiate the surface, thus inducing cleavage

only on the desired spots of the DMA. To demonstrate this, we prepared a droplet microarray with the format of $28 \times 96 = 2688$ square spots in the size of 500 μm and 250 μm borders and modified all the HL spots with HEPL. A quartz glass photo-masked with the logos of KIT and our group was put on top, and the surface was irradiated at 365 nm for 15 min with the intensity of 2.5 mW cm^{-2} . During the UV-triggered cleavage process, the nitro-substituent of the linker HEPL is reduced to a nitroso group (c-HEPL, Figure 2A), which leads to a bathochromic shift and an occurring yellow color. The irradiated areas showed the respective yellow logos (Figure 2C, top). When a DMA with $5 \times 16 = 80$ round spots with a diameter of 3 mm was irradiated in the same way, but with a chess pattern photomask, the developed color was clearly seen without any adjustments (Figure 2C, bottom). The controlled irradiation and cleavage give an additional dimension for variation of screening conditions on the droplet microarray. Our results show that the release of compounds can not only be controlled quantitatively via irradiation time but also spatially via selected irradiation of the HL spots.

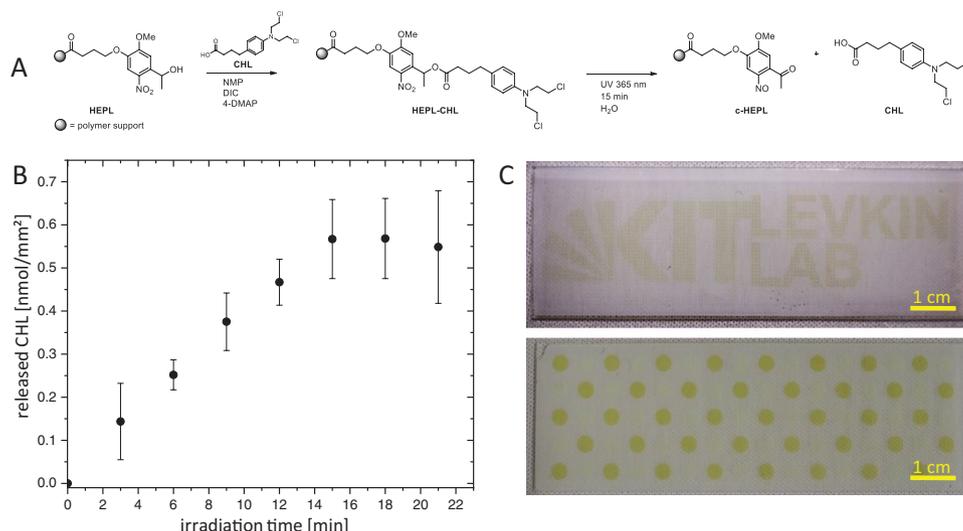


Figure 2. A) Attachment of CHL onto the HEPL with subsequent release upon UV-irradiation, leaving the cleaved photolinker (c-HEPL) on the surface. B) Kinetics of the UV-triggered release of CHL as a model drug from the surface into the 5 μ L droplets, determined by the spectroscopic analysis of the solution after different irradiation times. Each data point is the average of three experiments. Error bars represent standard deviation. C) Spatial control of cleavage of HEPL via UV light. During cleavage of the linker, a yellow color is developed. Droplet microarrays were prepared and modified with HEPL in all HL spots prior to irradiation through photomasks. Top pattern: 2688 square spots with 500 μ m edge length and 250 μ m borders. “KIT Levkin Lab” logo was used as inverted photomask. Bottom pattern: 80 round spots with 3 mm in diameter were irradiated in a checker-board pattern. UV linker HEPL gives full control over spatial release of compounds.

The surface chemistry was analyzed by the Raman microscopy. For this, we prepared three samples according to the standard protocol (Figure 3): a DMA slide with free amino groups of the immobilized cysteamine and HEPL-modified slides before and after UV-irradiation. For the first sample (Figure 3A), the spectrum shows bands at 2931 and 1440 cm^{-1} for the C–H bonds of the polymethacrylate substrate, 1732 cm^{-1} for the C=O stretching vibration, and at 3221 cm^{-1} for the polymers hydroxyl groups and 2120 cm^{-1} for alkyne groups which remained in the polymer from the production process. Since alkyne groups give a very strong signal in Raman spectroscopy, even residual amounts are detected and visible in the spectra. When the DMA is modified with the aromatic nitro group containing linker (Figure 3B), bands at 1333 and 1579 cm^{-1} are evolving. These bands correlate with the vibrations of C–(NO₂) and C–C (aromatic) and indicate the presence of the linker on the surface. Additionally, the band at 1276 cm^{-1} can be assigned to the linker’s hydroxyl group. After irradiating this spot with UV light (365 nm, 2.5 mW cm^{-2} , 15 min, Figure 3C), the intensities of the bands representing the nitro group and the benzylic alcohol decrease again, indicating the reductive transformation of the nitro group to a nitroso group and the oxidation of alcohol to carbonyl during the UV-induced cleavage. The bands of the polymer background are not affected by this transition.

Next, we assigned the band between 1250 and 1370 cm^{-1} to the nitro group of HEPL (Figure 3B) and a 2D mapping was achieved by measuring 81 single spectra on a field of ca. 400 \times 400 μ m (focusing on the edge of an HL spot). The resulting band intensities were plotted as a function of position (Figure 3D). Expectedly, the linker is only detected in the HL area, owing to the precision of the photolithographic patterning process.^[16]

2.3. Combinatorial Chemistry via Ugi Reaction

In order to prove the versatility of the DMA in employing multicomponent combinatorial chemistry, we performed the Ugi four-component reaction^[26] (Figure 4), which is widely used in combinatorial library synthesis.^[27] It merges four different starting materials: an amine, a carboxylic acid, an aldehyde or a ketone, and an isocyanide into one molecule with a characteristic bisamide structure. It offers four entry points for variation. The advantage of multicomponent reactions, as opposed to reactions with two reagents, is that significantly fewer variations of each building block are needed to create the same number of products. Thus, if every building block has 20 possible variations, it is possible to synthesize $20^4 = 160\,000$ different compounds. In order to synthesize the same number of compounds using a two-component reaction, it would require 400 variations of each of two reagents (400^2).

Table 1 shows all the educts used in this work. We used five different amino acids as surface-bound amines (1–5), three isocyanides (6–8), seven carboxylic acids (9–15), and eight carbonyls (16–23), which allow for a total of 840 unique products of the Ugi reaction. For each compound, a corresponding number of spots was functionalized with the linker and was used to synthesize a particular product. The combinatorial approach was realized by varying the starting materials in each spot. We first used a larger round spots with 3 mm diameter to investigate the reaction performance via liquid chromatography–mass spectrometry (LC-MS) and continued with 1 mm squares to show the possibility of miniaturization. By collecting the solution of five spots after cleavage, each with the same target compound, it was possible to have enough sample volume for LC-MS analysis of 13 exemplary reactions (Table 2, products 24–36), which showed purities between 21% and 91%. These

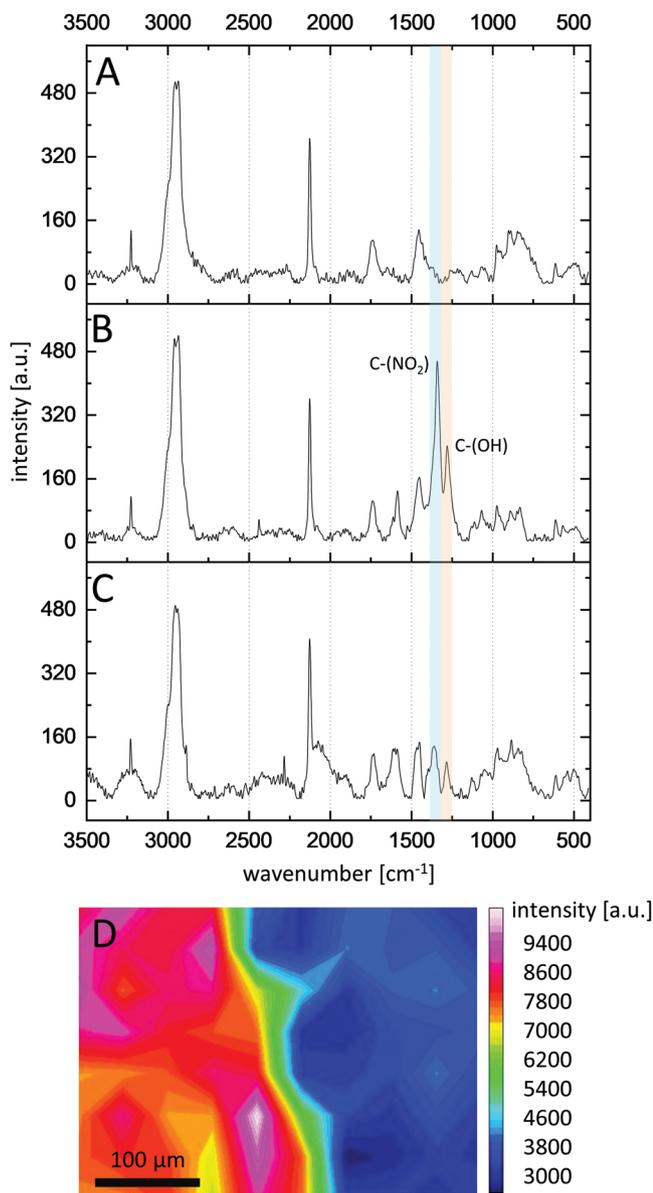


Figure 3. Raman analysis of a round HL spot on the DMA with a diameter of 3 mm. A) The free amine groups of the cysteamine are present in the HL spots after DMA production and before modification with HEPL. Intense bands of C-H (2931 cm^{-1}) and alkyne groups (2120 cm^{-1} , remaining on the surface) are visible from the polymer support. B) Attachment of HEPL is detectable by the increased band intensity at 1333 cm^{-1} referring to its nitro group and 1579 cm^{-1} for the aromatic system. C) UV irradiation (365 nm , 2.5 mW cm^{-2} , 15 min) of the surface reduces the nitro group of HEPL to nitroso and decreases these bands intensity. Additionally, the band at 1276 cm^{-1} of the benzylic alcohol is reduced due to its oxidation to the ketone. D) Raman microscopy mapping of the edge of a round spot ($d = 3\text{ mm}$) modified with HEPL. Colors show the intensity distribution of the band at $1250\text{--}1370\text{ cm}^{-1}$ corresponding to the C-(NO₂) vibration of the linker.

values are in the expectable range of Ugi reactions without fine tuning of the reaction conditions for every single combination of starting materials.^[28–30]

To prove the possibility of miniaturization on our platform, we switched from the round HL spots with a diameter of 3 mm, which could accommodate 5 μL to square spots

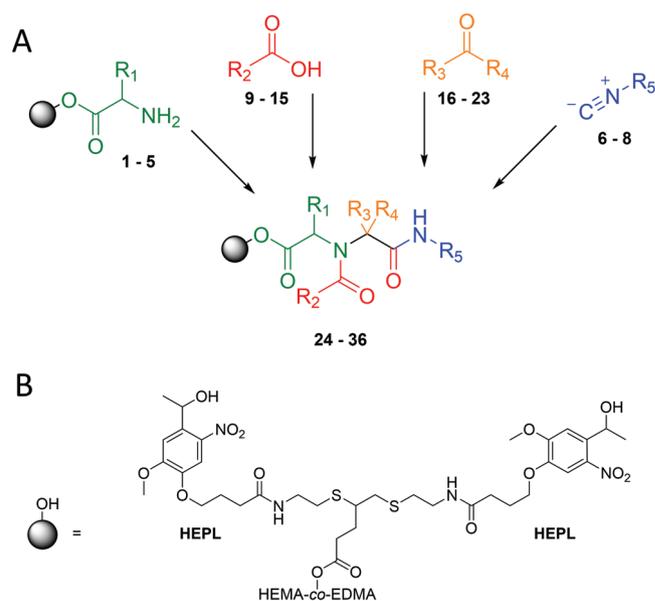
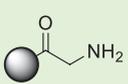
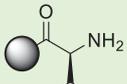
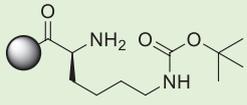
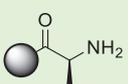
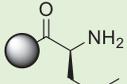
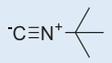
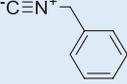
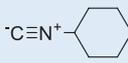
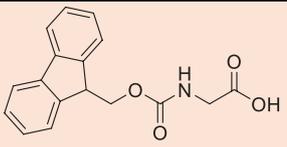
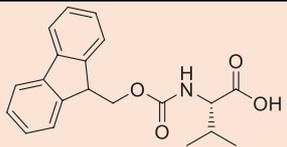
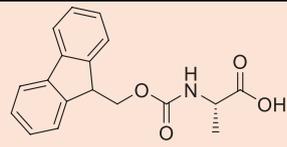
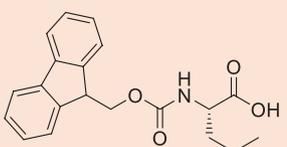
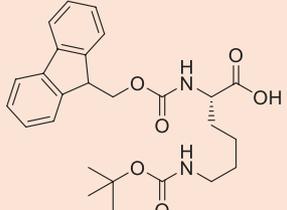
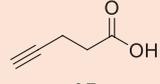
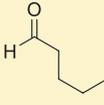
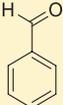
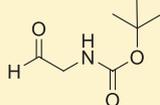
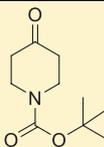
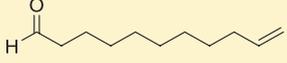


Figure 4. Scheme of the Ugi four-component reaction with variable entries for surface-anchored amines (1–5), carboxylic acids (9–15), aldehydes or ketones (16–23) and isocyanides (6–8), and the formed bisamides (products 24–36). Bottom: a detailed representation of the solid-phase chemistry. Each 4-pentynoic acid tethered to the polymer layer acts as a branching point and bears two linker molecules, effectively doubling the overall number of reaction sites.

with an edge length of 1 mm and volume of around 100 nL. This variation enlarged the library on a single DMA slide from 80 spots to 588 and the compound density from 4 to 30 spots cm^{-2} . Square-shaped spots use the available space on the slide more efficiently, leading to denser arrays. In every spot, 30 nL of the aldehyde or ketone solution was printed by a noncontact liquid dispenser and incubated in a closed Petri dish for 15 min to preform the imine. Subsequently, 35 μL of the carboxylic acid solution and 40 μL of the isocyanide solution per spot were printed to start the reaction. Isocyanide was added at last to avoid acid-induced hydrolysis. All solutions were used with a concentration of 0.5 M in GBL. The DMA was incubated for three days in the dark at room temperature in a closed Petri dish with a GBL-soaked paper tissue to avoid evaporation. To end the reaction, the reaction mixtures were washed from the DMA with acetone and ethanol and the slide was dried in air flow. 100 nL of deionized water was printed into each spot, and the products were released into the separated droplets by irradiating the whole DMA with UV light of 365 nm (2.5 mW cm^{-2}) for 15 min. MALDI matrix addition was performed by dispensing 100 nL per spot of a saturated solution of α -cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) in 10 vol% acetonitrile in water (containing $5 \times 10^{-3}\text{ M HCl}$).^[31] Directly after dispensing, the array was sandwiched onto an indium tin oxide (ITO)-coated glass slide with the same size as the DMA. The design of the sandwiching device that helps to align two slides over each other was reported previously.^[10] After contact of the droplets from the DMA with the ITO slide, the slides were separated again, leaving part of the droplets containing both synthesized compounds and matrix solution attached to the ITO surface in the array format

Table 1. Overview of the starting molecules for the Ugi reaction. Amines (1–5) are attached to the surface via the photolinker, while isocyanides (6–8), carboxylic acids (9–15), and carbonyls (16–23) are added as 0.5 M solutions in GBL. These 23 compounds can lead to 840 different products of the Ugi reaction.

 1	 2	 3
 4	 5	
 6	 7	 8
 9	 10	 11
 12	 13	 14
		 15
 16	 17	 18
 19	 20	 21
 22	 23	

mirroring the DMA slide. Due to inhomogeneities of the surface, the dispensing process and droplet evaporation, some droplets were smaller and could not come in contact with the ITO slide. Additional 20 nL of matrix mixture was added to those droplets and the sandwiching was repeated until all droplets were in contact with the ITO slide. This process copies

the array of single droplets, each with a synthesized product, from the DMA to the ITO slide. ITO slides are conductive and are commonly used for MALDI imaging. In preliminary experiments, we used standard MALDI steel plates, which also worked. Nevertheless, the transparency of the ITO slides simplifies the handling during the sandwiching because it is

Table 2. Purity of 13 exemplary compounds synthesized through the on-chip Ugi reaction as determined by LC-MS.

Product	Amine	Isocyanide	Carboxylic acid	Carbonyl	Purity ^{a)}
24	1	6	14	21	91%
25	1	7	10	16	58%
26	1	7	11	16	57%
27	1	7	12	16	43%
28	1	7	13	16	29%
29	1	7	15	16	21%
30	4	7	9	19	65% ^{b)}
31	4	7	9	23	59% ^{b)}
32	4	7	9	21	35% ^{b)}
33	1	8	9	16	23%
34	3	8	9	16	21%
35	4	8	9	16	24%
36	5	8	9	16	32%

^{a)}Purity was calculated via LC-MS analysis of the cleavage solution as the product peaks ratio of summarized peak area at 254 nm absorbance.; ^{b)}The peaks of the two formed diastereomers were combined.

visible when the droplets are in contact with the ITO surface. The deposited mixtures were dried in vacuum and passed to MALDI-TOF analysis. As it can be seen in **Figure 5E**, the obtained spectrum of representative compound **37** gives the $[M+Na]^+$ - adduct peak with the visible isotopic pattern.

2.4. Proof-of-Principle Cell Experiments

To demonstrate the performance of the DMA as a platform for the solid-phase synthesis that can be directly followed by a biological read-out or a cell assay, we used CHL (see **Figure 2A**) as a model bioactive compound. The drug was attached to the linker in the HL spots via Steglich esterification (5 μ L per spot of NMP containing 50×10^{-3} M CHL hydrochloride, 50×10^{-3} M diisopropyl carbodiimide (DIC), and 5×10^{-3} M 4-(dimethylamino)pyridine (4-DMAP) over 4 h); the slide was subsequently washed and sterilized in 70 vol% ethanol/water. To prove that CHL is not physically adsorbed, but covalently bound to the surface and can be only released upon UV irradiation, CHO-K1 cells were seeded onto two slides functionalized with CHL. One slide was exposed to the UV light for 20 min, the other slide was not exposed to the UV light and served as a control. CHO-K1 cells were also seeded on control slides with free hydroxyl groups (coming from modification with β -mercaptoethanol) and one of them was exposed to the UV light for 20 min to investigate the effect of UV light alone on cell viability. Subsequently, cells were cultivated for 24 h and stained with CalceinAM and propidium iodide (PI) for the live/dead analysis. The results of the fluorescence microscopy (**Figure 6** and **Figure S2**, Supporting Information) show that only after exposing the CHL-functionalized surface to UV light, cell viability is decreased. On the negative control slide (not CHL-modified and not UV irradiated), the cell viability was found to be 97% and there was no significant effect of UV irradiation on the viability (96%). Modification of DMA

with CHL did not reduce the viability of the cells, provided the slides were not irradiated with UV light. The combination of the CHL pre-modification with UV irradiation, however, led to a significant drop of the viability to 3%, confirming the release of CHL into the droplets. This experiment proves that cells' viability (as measured by staining with CalceinAM and PI and subsequent fluorescence microscopy) is neither affected by the photo cleavage conditions (365 nm, 20 min, 2.5 mW cm^{-2}) for the drug release nor by the surface-bound drug alone. This result suggests that it is possible to "store" a drug in the reservoir of the polymer layer in the HL spot until it has to be released into the droplet microreservoir. It also allows us to have a low cell-to-drug contact area while still having enough sample to obtain an active concentration after photorelease.^[32] With a loading of CHL of 0.68 nmol mm^{-2} on 3 mm round spots and a spot volume of 5 μ L, the maximum achievable concentration is 954×10^{-6} M. By thickening the polymer layer, the loading can be increased with the drawback of a higher background signal during fluorescence microscopy. The coatings in this work were around 12 μ m thick as measured by optical profilometry and offer a suitable balance between loading capacity and requirements for the microscopy readout.

3. Conclusion

We present a new method for miniaturized on-chip combinatorial synthesis and screening by using the droplet microarray as support for solid-phase synthesis, followed by a screening of cells in individual nanoliter droplets, thereby drastically reducing reagent and cell consumption.^[33] A photocleavable linker was introduced as an anchor point for the starting material of the synthesis and 13 exemplary compounds were synthesized via Ugi four-component reaction in 3 mm round spots with volumes of 5 μ L. The synthesis was then further miniaturized down to 1 mm HL squares to achieve higher density and volumes of 100 nL per reaction. A library of 588 bisamide Ugi products was constructed on a single chip. After the synthesis, the chip could be washed to remove excess reaction solution, and the product compounds were photoreleased into distinct droplets. The array format of the droplet microarray allowed us to copy the compounds onto an ITO slide via sandwiching to perform MALDI-MS analysis of the synthesized library. We then demonstrated the possibility to release compounds from the polymer matrix into nanoliter droplets and to control the released amounts by UV. We were able to spatially address single spots of the DMA via patterned irradiation, thus showing the possibility of various independent screening conditions on a single chip. Additionally, we used CHL as a model cytotoxic compound, which was attached to the HL spots through the photocleavable linker. UV-induced release of the drug was shown to significantly decrease cell viability in the droplets containing the drug, while cells were not affected by UV alone or by the irradiation of droplets without surface surface-bound CHL. Thanks to the arrayed format, the single reactions and cell experiments can be tracked or analyzed directly on the array in a time-lapse way. The presented photoreleasing droplet microarray platform will allow further miniaturization of high-throughput combinatorial library synthesis and its hyphenation with cell-based biological screenings.

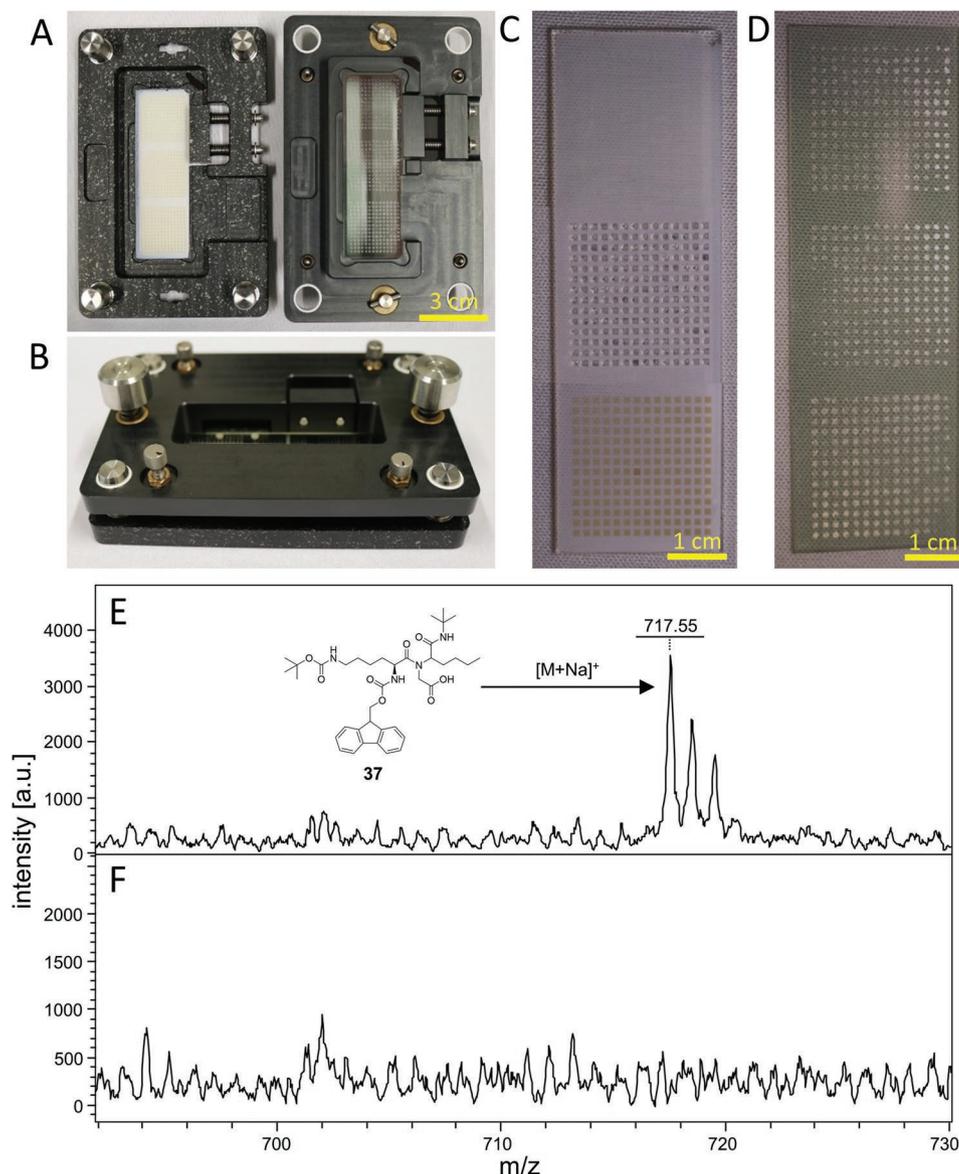


Figure 5. Process of copying the droplet microarray onto an ITO slide for MALDI-TOF MS measurements. A) The sandwiching device helps to align both slides onto each other. Left side is the bottom part with the irradiated droplet microarray while on the right is the top part with the ITO slide (already stamped with matrix). B) Closed sandwiching device. The two connectors on the sides pull the two halves together while the distance is controlled by four screws. Top part is lowered until all droplets touch the ITO slide. C) Merged photograph of a dry DMA (14 × 14 mm HL spots, top), the DMA with 100 nL water in each spot (middle) and the dry array after UV irradiation (bottom). D) ITO slide after copying the droplet microarray which contained matrix mixture (CHCA/DHB) in the droplets and drying in vacuum. E) MALDI-TOF spectra of compound **37** as sodium adduct ([M+Na]⁺, detected: 717.55 m/z, calc.: 717.38 u) and F) matrix background. The compound was first cleaved into distinct droplets via UV irradiation, followed by the addition of 100 nL of a saturated solution of CHCA and DHB matrix in 10% ACN/water (containing 5 × 10⁻³ M HCl) into each spot and sandwiching the DMA slide onto an ITO slide to copy the array. The ITO slide was dried in vacuum and analyzed by MALDI-MS.

4. Experimental Section

Materials: The glass slides were purchased from Schott Nexterion (Jena, Germany). ITO slides were purchased from Diamond Coatings (Halesowen, United Kingdom) and immersed in 3 wt% hydrogen peroxide for 10 min prior to use.

4-Pentynoic acid was purchased from Apollo Scientific (Bredbury, UK). Fmoc-Gly-OH was purchased from Bachem (Bubendorf, Switzerland). Fmoc-Val-OH, Fmoc-Ala-OH and Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH were purchased from Iris Biotech (Marktredwitz, Germany). Hydrochloric acid (37 %), ethanol, acetone, 4-DMAP, HEPL pyridine, methanol, DMF, NMP, acetic acid (glacial), acetic acid

anhydride, dichloromethane, propylamine, piperidine, cyclohexanone, pentanal, benzaldehyde, *N*-Boc-aminoacetaldehyde, *N*-Boc-piperidone, 10-undecenal, hydrogen peroxide (30 w%), formaldehyde (37 w%), GBL, and Ham's F12 medium were purchased from Merck (Darmstadt, Germany). 1-Hydroxybenzotriazole was purchased from Molekula (Newcastle upon Tyne, UK). Sodium hydroxide, 3-(trimethoxysilyl)propyl methacrylate, 2-hydroxyethyl methacrylate, ethylene dimethacrylate, 1-decanol, cyclohexanol, 2,2-dimethoxy-2-phenylacetophenone, 1*H*,1*H*,2*H*,2*H*-perfluorodecanethiole, cysteaminium chloride, *tert*-butyl isocyanide, cyclohexyl isocyanide, benzyl isocyanide, CHL were purchased from Sigma-Aldrich (St. Louis, USA). CalceinAM and Trypsin were purchased from Life Technologies (Darmstadt, Germany), PI

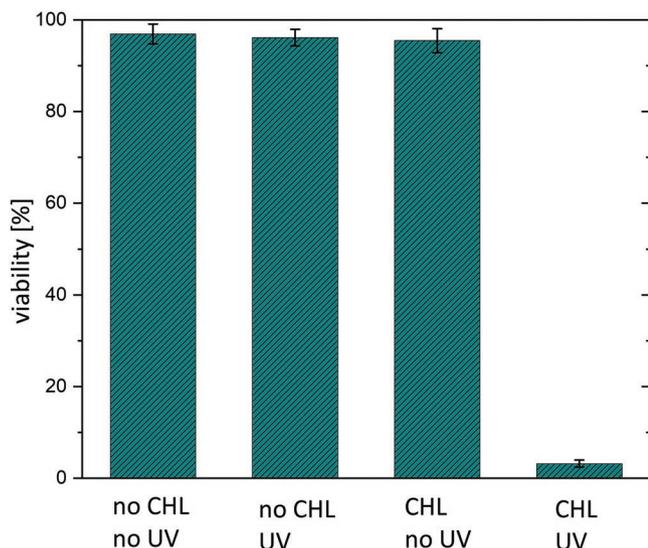


Figure 6. CHO-K1 cells were seeded on the DMA and their viability was determined via CalceinAM/PI staining for differently treated surfaces after incubation time of 24 h. Viability was not affected either by UV light or by the presence of the immobilized CHL. The cell viability was decreased only after irradiation the surface with attached CHL, which led to the release of the drug into the droplets. Every bar represents an average of three repetitions of the same experiment. Error bars represent the standard deviation.

was purchased from Invitrogen (Merelbeke, Belgium). If not stated otherwise, all chemicals have been used without further purification.

Automated liquid dispensing was performed by iDot (Dispendix, Stuttgart, Germany).

Intensity of the UV light source was measured by a UV-Meter μ C Basic (Hönle, Gräfelfing, Germany) using the UV-A sensor.

In Figure 2C, the top picture was adjusted by increasing the contrast in Microsoft PowerPoint to improve visibility of the logo.

Functionalization of the Polymer with Hydroxyethyl Photolinker. The DMA-slides were prepared according to the previously published procedure.^[11,33] Briefly, a microscope glass slide ($76 \times 26 \times 1$ mm) was coated with a layer of nanoporous HEMA-co-EDMA followed by a Steglich esterification of the polymer using 4-pentynoic acid. Next, the surface was wetted with a solution of 1H,1H,2H,2H-perfluorodecanethiols in isopropanol and covered with a quartz photomask of the desired pattern. After UV irradiation at 254 nm (2.0 mW cm^{-2}) for 2 min, the slide was washed, a solution of cysteaminium chloride in 1:1 EtOH/water was spread, and it was covered with a clear quartz glass slide before irradiating it again with UV light.

To immobilize the linker onto the surface of the HL spots, HEPL, DIC, and 1-hydroxybenzotriazol were mixed in NMP to a final concentration of 0.1 M per compound. To a round spot ($d = 3$ mm), 5 μ L of this solution was added and incubated in the dark at room temperature for 18 h. Respective volume for the square spots ($b = 1$ mm) was 100 nL. The DMA was then washed with acetone and dried in nitrogen flow.

Attachment of CHL: After the linker attachment, each spot was filled with 5 μ L of NMP containing 50×10^{-3} M CHL hydrochloride, 50×10^{-3} M DIC, and 5×10^{-3} M 4-DMAP. The DMA was left in the dark for 8 h at room temperature, subsequently rinsed with acetone and dried in nitrogen flow.

Photorelease from the Surface: If not stated otherwise, the UV-induced cleavage from the surface was performed by pipetting 5 μ L of deionized water into each round spot ($d = 3$ mm) and irradiating the DMA in a Biolink BLX UV chamber (Witec, Sursee, Switzerland) with 2.5 mW cm^{-2} at 365 nm for 15 min. The aqueous solutions were then collected into glass vials and 5 μ L DMF was applied into each spot to dissolve

potential hydrophobic compounds. The DMF was then combined with the aqueous phases of the corresponding spots.

Photorelease Kinetics Measurements: A CHL-modified DMA was irradiated for different times. After irradiation, the volume of four spots (40 μ L in total) was pipetted off the surface and diluted to 100 μ L with DMF/water (1:1). This solution was then measured for absorbance at 284 nm. To calculate the concentration of CHL in a droplet, a calibration curve was constructed. 16.5 mg CHL was dissolved in 10 mL DMF/water (1:1). This stock solution was then diluted 1:10 three times, and absorbance of every dilution was measured at 284 nm with path lengths of 10 and 2 mm.

Raman Microscopy: The used UV/VIS spectrometer was a Lambda 35 by PerkinElmer (Waltham, USA). Raman spectra and 2D microscopy were performed on a Bruker Senterra instrument (Ettlingen, Germany). Illumination was executed by a laser beam at 532 nm and 2 mW intensity. 2D mapping was achieved by measuring 81 spectra over an area of $400 \mu\text{m} \times 400 \mu\text{m}$ and plotting the intensity of a selected band over the sampled area.

Ugi Reaction: The amino acids were loaded onto the linker in the HL spots by adding 5 μ L (for 3 mm spots; 100 nL for 1 mm) of a solution of NMP containing 100×10^{-3} M of the Fmoc-protected amino acid, 100×10^{-3} M DIC, and 10×10^{-3} M 4-DMAP in the dark at room temperature overnight (18 h). After washing the slide with acetone, the Fmoc group was removed by the addition of 5 μ L of 20% piperidine in NMP and incubation for 20 min. This process was repeated once after washing off the first solution. In each round spot ($d = 3$ mm), 2 μ L of the aldehyde or ketone solution (0.5 M in GBL) was pipetted and equilibrated for 15 min to preform the imine. Subsequently, 2.5 μ L of carboxylic acid (0.5 M in GBL) and 3 μ L of isocyanide solution (0.5 M in GBL) was added which summed up to 7.5 μ L reaction mixture per spot. For the square spots ($b = 1$ mm), the volumes were 30 nL amine, 35 nL acid, and 40 nL isocyanide, and a contactless liquid dispenser (iDOT) was used. The slide was incubated for 62 h at room temperature in a closed and darkened Petri dish with a GBL-soaked paper tissue to minimize evaporation. Afterward, the slide was extensively washed with acetone and dried in nitrogen flow.

MALDI-TOF, LC/MS, and Electrospray Ionization (ESI)-MS Spectrometry: MALDI-TOF spectra were obtained on an Autoflex III (Bruker, Bremen, Germany) in the positive reflector mode.

MicrOTOF-Q II (Bruker) was used for ESI-MS analysis and an HP 1100 (Hewlett-Packard, Palo Alto, USA) system with a C18 column (XB-C18, 100 \AA , 100×4.6 mm, Phenomenex, Torrance, USA) was used for LC-MS analysis of products. Mobile phase contained acetonitrile (0.1% formic acid) as A and deionized water (0.1% formic acid) as B with a gradient of A from 10% to 99% over 10 min, remaining for 5 min and decreasing back to 10% over 5 min. Spectra were analyzed via "Spectrus Processor" software (Advanced Chemistry Development, Toronto, Canada).

Cell Experiments: Chinese hamster ovary (CHO-K1, ECACC 85051005) cells were provided by ScreenFect (Eggenstein-Leopoldshafen, Germany). Cells were cultured in Ham's F12 medium with 10% fetal calf serum in a humid incubator at 37 $^{\circ}\text{C}$ with 5% CO_2 . They were passaged every 2 to 3 days by detachment with 0.25% trypsin/ethylenediaminetetraacetic acid solution and dilution in fresh media. All biological experiments were performed under sterile conditions in a clean bench with a laminar airflow.

A DMA with linker-bound CHL was used as a positive control, while a DMA which was functionalized with 2-mercaptoethanol in the HL spots served as negative control. All slides, which were used for cell tests, were sterilized in 70 vol% ethanol in water prior to use. A 2.2% w/w gelatin solution was prepared by adding 3 mL of sterile cell medium to 66 mg gelatin from bovine skin. To increase the solubility of gelatin, the solution was gently warmed in a water bath at 37 $^{\circ}\text{C}$. Once gelatin was completely dissolved, the solution was sterilized by filtering through sterile 0.22 μm filter. The gelatin solution was then applied onto all DMA spots via rolling droplet to produce evenly distributed droplets. The DMA was then incubated for 30 min at 37 $^{\circ}\text{C}$ and 5% CO_2 , followed by air drying of gelatin under sterile conditions. 5 μ L of CHO-K1 cell suspension was

seeded onto the spots with a concentration of 1×10^5 cells mL^{-1} , leading to around 500 cells per spot. One DMA functionalized with CHL and one DMA with β -mercaptoethanol only (serving as a control) were irradiated with UV-light at 365 nm. Cells were incubated for 24 h at 37 °C and 5% CO_2 , stained with CalceinAM/PI and counted manually to calculate cell viability. Viability was calculated as

$$\text{Viability}[\%] = N_{\text{CalceinAM}} / (N_{\text{CalceinAM}} + N_{\text{PI}}) \times 100 \quad (1)$$

where $N_{\text{CalceinAM}}$ is the number of cells stained with CalceinAM and N_{PI} is the number of cells stained with PI.

Image Acquisition: Microscopic images were taken using a Keyence BZ-9000 microscope (KEYENCE, Osaka, Japan) with a mercury vapor lamp as light source and the following filters for fluorescence imaging: OP-79301 SB Filter GFP-BP and OP-79302 SB Filter TexasRed. Exposure times were set manually for every experiment to achieve good contrast. Images were taken using a 10x objective.

Supporting Information

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

Acknowledgements

This project was supported by ERC Starting Grant (DropCellArray, 337077) and ERC Proof-of-Concept Grant (CellScreenChip, 680913). The authors thank the Compound Platform (DFG project 284178167) for support with LC-MS analysis and Dr. Olaf Fuhr (KIT, Institute of Nanotechnology) for training and access to ESI-MS.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

high throughput, nanomolar, photorelease, screening, solid-phase synthesis

Received: October 17, 2019

Revised: December 4, 2019

Published online: January 27, 2020

- [1] J. A. DiMasi, H. G. Grabowski, R. W. Hansen, *J. Health Econ.* **2016**, *47*, 20.
- [2] R. Frank, *J. Immunol. Methods* **2002**, *267*, 13.
- [3] H. E. Blackwell, *Curr. Opin. Chem. Biol.* **2006**, *10*, 203.
- [4] F. Deiss, W. L. Matochko, N. Govindasamy, E. Y. Lin, R. Derda, *Angew. Chem., Int. Ed.* **2014**, *53*, 6374.
- [5] P. M. López-Pérez, E. Grimsey, L. Bourne, R. Mikut, K. Hilpert, *Front. Chem.* **2017**, *5*, 25.
- [6] A. Kramer, T. Keitel, K. Winkler, W. Stöcklein, W. Höhne, J. Schneider-Mergener, *Cell* **1997**, *91*, 799.
- [7] K. Hilpert, D. F. H. Winkler, R. E. W. Hancock, *Nat. Protoc.* **2007**, *2*, 1333.
- [8] M. Beyer, A. Nesterov, I. Block, K. König, T. Felgenhauer, S. Fernandez, K. Leibe, G. Torralba, M. Hausmann, U. Trunk, V. Lindenstruth, F. R. Bischoff, V. Stadler, F. Breitling, *Science* **2007**, *318*, 1888.
- [9] V. Stadler, T. Felgenhauer, M. Beyer, S. Fernandez, K. Leibe, S. Güttler, M. Gröning, K. König, G. Torralba, M. Hausmann, V. Lindenstruth, A. Nesterov, I. Block, R. Pipkorn, A. Poustka, F. R. Bischoff, F. Breitling, *Angew. Chem., Int. Ed.* **2008**, *47*, 7132.
- [10] A. A. Popova, C. Depew, K. M. Permana, A. Trubitsyn, R. Peravali, J. Á. G. Ordiano, M. Reischl, P. A. Levkin, *SLAS Technol.* **2017**, *22*, 163.
- [11] A. A. Popova, S. M. Schillo, K. Demir, E. Ueda, A. Nesterov-Mueller, P. A. Levkin, *Adv. Mater.* **2015**, *27*, 5217.
- [12] M. Benz, M. R. Molla, A. Böser, A. Rosenfeld, P. A. Levkin, *Nat. Commun.* **2019**, *10*, 2879.
- [13] A. Rosenfeld, M. Brehm, A. Welle, V. Trouillet, S. Heissler, M. Benz, P. A. Levkin, *Mater. Today Bio* **2019**, *3*, 100022.
- [14] C. Hulme, V. Gore, *Curr. Med. Chem.* **2003**, *10*, 51.
- [15] J. E. Biggs-Houck, A. Younai, J. T. Shaw, *Curr. Opin. Chem. Biol.* **2010**, *14*, 371.
- [16] W. Feng, L. Li, E. Ueda, J. Li, S. Heißler, A. Welle, O. Trapp, P. A. Levkin, *Adv. Mater. Interfaces* **2014**, *1*, 1400269.
- [17] E. Ueda, F. L. Geyer, V. Nedashkivska, P. A. Levkin, *Lab Chip* **2012**, *12*, 5218.
- [18] R. J. Jackman, D. C. Duffy, E. Ostuni, N. D. Willmore, G. M. Whitesides, *Anal. Chem.* **1998**, *70*, 2280.
- [19] C. P. Holmes, *J. Org. Chem.* **1997**, *62*, 2370.
- [20] K. Usui, T. Kikuchi, K.-Y. Tomizaki, T. Kakiyama, H. Mihara, *Chem. Commun.* **2013**, *49*, 6394.
- [21] H. A. Kenny, M. Lal-Nag, E. A. White, M. Shen, C.-Y. Chiang, A. K. Mitra, Y. Zhang, M. Curtis, E. M. Schryver, S. Bettis, A. Jadhav, M. B. Boxer, Z. Li, M. Ferrer, E. Lengyel, *Nat. Commun.* **2015**, *6*, 6220.
- [22] M. H. Beresini, Y. Liu, T. D. Dawes, K. R. Clark, L. Orren, S. Schmidt, R. Turincio, S. W. Jones, R. A. Rodriguez, P. Thana, D. Hascall, D. P. Gross, N. J. Skelton, *J. Biomol. Screening* **2014**, *19*, 758.
- [23] B. C. Duffy, L. Zhu, H. Decornez, D. B. Kitchen, *Bioorg. Med. Chem.* **2012**, *20*, 5324.
- [24] A. Kramer, U. Reineke, L. Dong, B. Hoffmann, U. Hoffmüller, D. Winkler, R. Volkmer-engert, J. Schneider-mergener, *J. Pept. Res.* **1999**, *54*, 319.
- [25] T. Ast, N. Heine, L. Germeroth, J. Schneider-Mergener, H. Wenschuh, *Tetrahedron Lett.* **1999**, *40*, 4317.
- [26] I. Ugi, *Angew. Chem., Int. Ed. Engl.* **1962**, *1*, 8.
- [27] I. Akritopoulou-Zanze, *Curr. Opin. Chem. Biol.* **2008**, *12*, 324.
- [28] J.-C. Bradley, K. B. Mirza, T. Osborne, A. Williams, K. Owens, *J. Visualized Exp.* **2008**, *21*, 942.
- [29] S. Marcaccini, T. Torroba, *Nat. Protoc.* **2007**, *2*, 632.
- [30] A. Dömling, I. Ugi, *Angew. Chem.* **2000**, *39*, 3168.
- [31] S. Laugesen, P. Roepstorff, *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 992.
- [32] C. N. Robson, J. Alexander, A. L. Harris, I. D. Hickson, *Cancer Res.* **1986**, *46*, 6290.
- [33] A. A. Popova, K. Demir, T. G. Hartanto, E. Schmitt, P. A. Levkin, *RSC Adv.* **2016**, *6*, 38263.