Supplementary Information for:

Peptide Array Functionalization *via* the Ugi Four-Component Reaction

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1. Methods

1.1. Materials

Chemicals and solvents from the following suppliers were used: Fmoc-β-Ala-OH, 4'-{(R,S)-alpha-[1-(9-Fluorenyl)methoxycarbonylamino]-2,4-dimethoxybenzyl}-phenoxyacetic acid (Fmoc-Rink-amide linker) and OxymaPure from Iris Biotech (Marktredwitz/ Germany); resin (S-LEC-P LT 7552) from Sekisui Chemical Co. Ltd. (Osaka/ Japan); DyLight 680-NHS and 5-(6)-carboxytetramethylrhodamine *N*-succinimidyl ester (TAMRA-NHS) from ThermoFisher Scientific (Waltham, USA); dry methanol (MeOH) and 2,2'-bipyridine from Alfa Aesar (Karlsruhe/ Germany); *N*,*N*,*N*',*N*'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, 4 Å molar sieve, dichloromethane (DCM), *N,N*-diisopropylethylamine (DIPEA), *N,N*-dimethylformamide (DMF), *N,N*-dimethylacetamide (DMAc), Methanol (MeOH), isopropanol, ethyl acetate, benzylamine and piperidine from Merck (Darmstadt/ Germany); trifluoroacetic acid (TFA), triisobutyl silane (TIBS), acetic anhydride, phosphate buffered saline (PBS), Tween20, Fmoc-Gly-OPfp, Fmoc-Lys(Boc)-OPfp, pentafluorophenol, hydroxybenzotriazole, copper(I)bromide, potassium hydroxide (KOH), poly(ethylene glycol) methyl methacrylate (average M_n 360), *N,N'*-diisopropylcarbodiimide (DIC), 1-methylimidazole, (3-aminopropyl) triethoxysilane (APTES), ethanol (EtOH), hexanal, isovaleraldehyde, cyclohexyl isocyanide, *tert*-butyl isocyanide, 10-undecenal, bicyclo[2.2.1] hept-5-ene-2-carboxaldehyde, hydroxybenzotriazole, *n*-butylformate, triethylamine (TEA), phosphoryl chloride and *α*-bromoisobutyryl bromide from Sigma Aldrich Chemie GmbH (Steinheim/ Germany). All used solvents were of analytical purity grade, except for the synthesis of the benzyl isocyanide: xylene, DCM, *n*-hexane, DCM and ethyl acetate were used as technical grade. DCM (dry) and DMF (dry) were stored above 4 Å molar sieve.

Polyimide Kapton foil was bought from CMC Klebetechnik GmbH (Frankenthal/ Germany).

The following synthesis surfaces were obtained from PEPperPRINT GmbH (Heidelberg/ Germany): All 10:90 poly(ethylene glycol) methacrylate-co-methyl methacrylate (PEGMA-co-MMA) synthesis slides functionalized with one Fmoc-β-Ala. Arrays carrying a β-Ala-Asp-β-Ala spacer further functionalized with alternating lysine and glycine spots.

Ultrapure water was produced with a Synergy® Water Purification System equipped with a Synergy Pak 2 from Merck (Darmstadt / Germany).

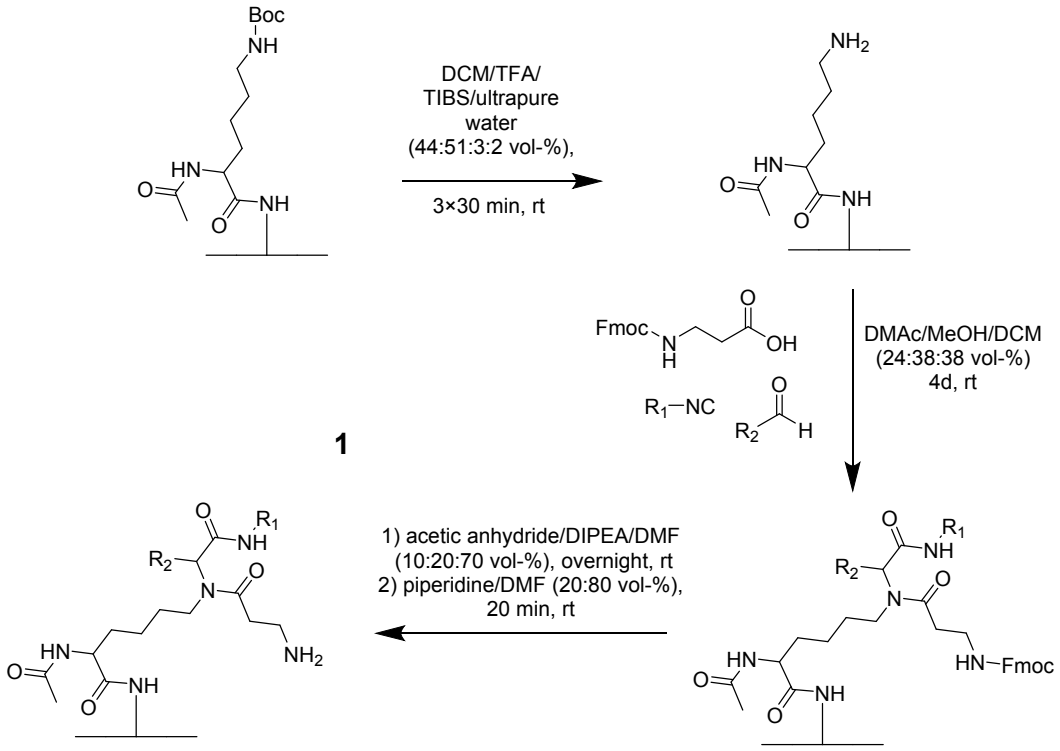
1.2. Experimental procedures

If not stated otherwise all reactions and washing steps with synthesis surfaces were performed with 10 mL of solvent.

1.2.1. Side chain deprotection

For arrays functionalized with Ac-Lys(Boc), the side-chain protecting group was removed by rocking the arrays in a solution, freshly prepared each time, of DCM/ TFA/ TIBS/ ultrapure water (44:51:3:2 % v/v)) for 3 × 30 min. Then, the slides were washed 2 × 5 min with DCM, 1 × 5 min with DMF, 1 × 30 min DIPEA/ DMF (5:95 % v/v), 2 × 5 min DMF and 2 × 3 min MeOH. Finally, the slides were dried under a stream of argon.[1]

1.2.2. The Ugi four-component reaction on synthesis surfaces



S. Scheme. 1: Reaction scheme of the array side chain deprotection, U-4CR, capping and Fmoc-deprotection.

Side-chain deprotected PEGMA-co-MMA arrays carrying alternating Lys and Gly spots or Fmoc deprotected slides with a spacer were immersed in 5 mL of a solution of 0.6 M Fmoc-β-Ala-OH, isocyanide (0.6 M for cyclohexyl isocyanide and benzyl isocyanide, 0.4 M for *tert*-butyl isocyanide) and aldehyde (0.6 M for hexanal and 0.4 M for isovaleraldehyde, 10-undecenal or bicyclo[2.2.1] hept-5-ene-2-carboxaldehyde) in DMAc/ DCM (dry)/ MeOH (dry) (24:38:38 % v/v) under argon atmosphere for 4 days. Subsequently, the slides were washed with DMF 5 × 5 min and capped directly afterwards.

1.2.3. Capping procedure

To cap the unreacted free amine groups on the surface, an acetylation was performed. Therefore, the slide was covered with a solution of acetic anhydride/ DIPEA/ DMF (dry) (10:20:70 % v/v) under argon atmosphere for 4 h (after functionalization with amino acids from solution) or overnight.

Afterwards, the slides were washed with DMF, 5 × 5 min and MeOH 2 × 3 min.[1,2] The capping step was followed by an Fmoc-deprotection step.

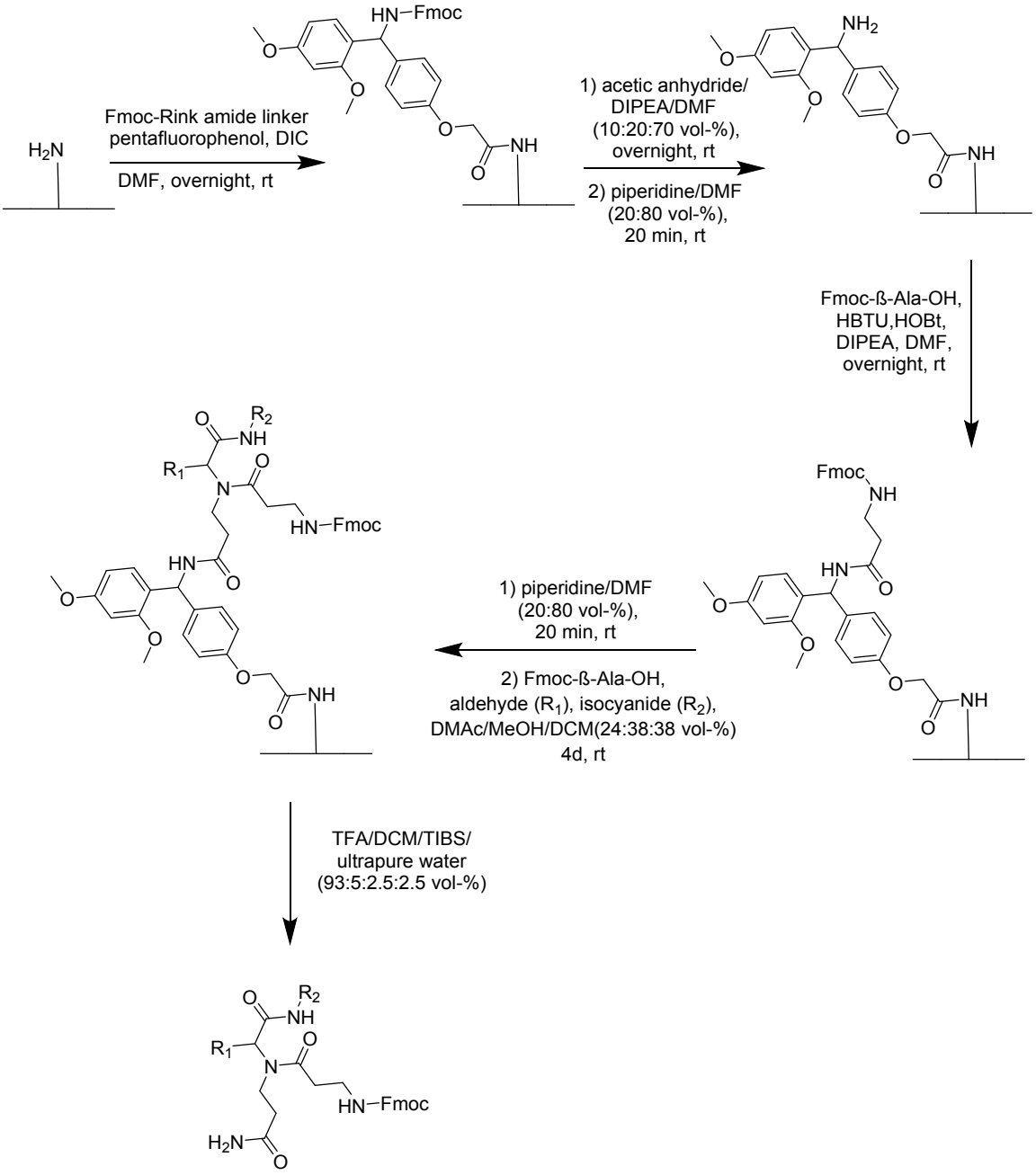
1.2.4. Fmoc-deprotection procedure

A PEGMA-co-MMA or pure poly(ethylene glycol) methacrylate (pure PEGMA) surface functionalized with a least one Fmoc-β-Ala or an Fmoc-protected growing peptide chain was deprotected by gently rocking the slide in a freshly prepared solution of piperidine/ DMF (dry) (20:80 % v/v) for 20 min. Subsequently, the slide was washed by rocking it in DMF 3 × 5 min followed by MeOH 2 × 3 min, each time the solvent was refreshed. Finally the slides were dried under a stream of argon.[1,2]

1.2.5. Fluorescent staining protocol

The capped and Fmoc-deprotected functionalized slides were pre-swollen with phosphate buffered saline with Tween20 (PBS-T) (500 µL per L) buffer for 10 min. Then, the slides were rocked in a solution of DyLight 680-NHS in PBS-T (1.00 µg per 10.0 mL) or TAMRA-NHS in PBS-T (1.00 µg per 10.0 mL) for 2 h. Subsequently, the slides were washed with PBS-T buffer for 5 min, with ultrapure water for 2 min, with DMF for 5 min, with ethyl acetate for 5 min and finally the slide was flushed with DCM and dried under a stream of argon.

1.2.6. Functionalization slides for MALDI-ToF measurements:



S. Scheme. 2: Schematic illustration of the reaction steps for functionalizing the surface with an Fmoc-Rink amide linker, which enables acidic detachment of the U-4CR reaction product from the surface for MALDI analysis.

A Fmoc-deprotected PEGMA-co-MMA slide (used for U-4CR employing of cyclohexyl isocyanide and hexanal and *tert*-butyl isocyanide and isovaleraldehyde) or a pure PEGMA slide (used for the U-4CR employing cyclohexyl isocyanide and 10-undecenal or bicyclo[2.2.1] hept-5-ene-2-carboxaldehyde) functionalized with one β-Ala was covered with 250 µL of a solution of 0.2 M Fmoc-Rink-amide linker, 0.2 M pentafluorophenol and 0.2 M DIC in DMF (dry) overnight.[1] The slide was washed with DMF 3 × 5 min, followed by a 4 h capping step. In the next step the slide was Fmoc-deprotected and covered with 250 µL of a solution of 0.2 M Fmoc-β-Ala-OH, 0.2 M *N*,*N*,*N*′,*N*′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, 0.2 M hydroxybenzotriazole or OxymaPure and 0.4 M DIPEA in DMF (dry) overnight. Afterwards the slide was washed with DMF 3 × 5 min and 2 × 3 min MeOH. Then, the slide was flushed with DCM and dried under a stream of argon. After Fmoc-

deprotection, an U-4CR reaction was performed under the same reaction conditions as stated before.

1.2.7. Removal of the Ugi product from the surface:

To detach the formed Ugi product, a PEGMA-co-MMA or a pure PEGMA synthesis surface functionalized with a Rink-amide linker followed by subsequent U-4CR was incubated with 1.00 mL of DCM for 10 min. Next, the DCM was removed and 1.00 mL of TFA/ DCM/ TIBS/ ultrapure water (92:3:2.5:2.5 vol-%) was added onto the surface, after 15 minutes additional solution was added onto the slide to avoid dryness and the surface was incubated for an additional 15 minutes. Finally, the surface was rinsed three times with 500 µL of DCM, followed by 500 µL of MeOH.[1] The incubation and rinsing solutions were combined and after evaporation of the solvent, the remaining crude product was investigated with MALDI-ToF.

1.2.8. Arrays produced with the laser-printing technique

The arrays functionalized with alternating Lys(Boc) and Gly spots were produced with the laser printing technique on a custom built laser printer by the commercial supplier PEPperPRINT.[3,4]

1.2.9. Combinatorial laser-induced forward transfer

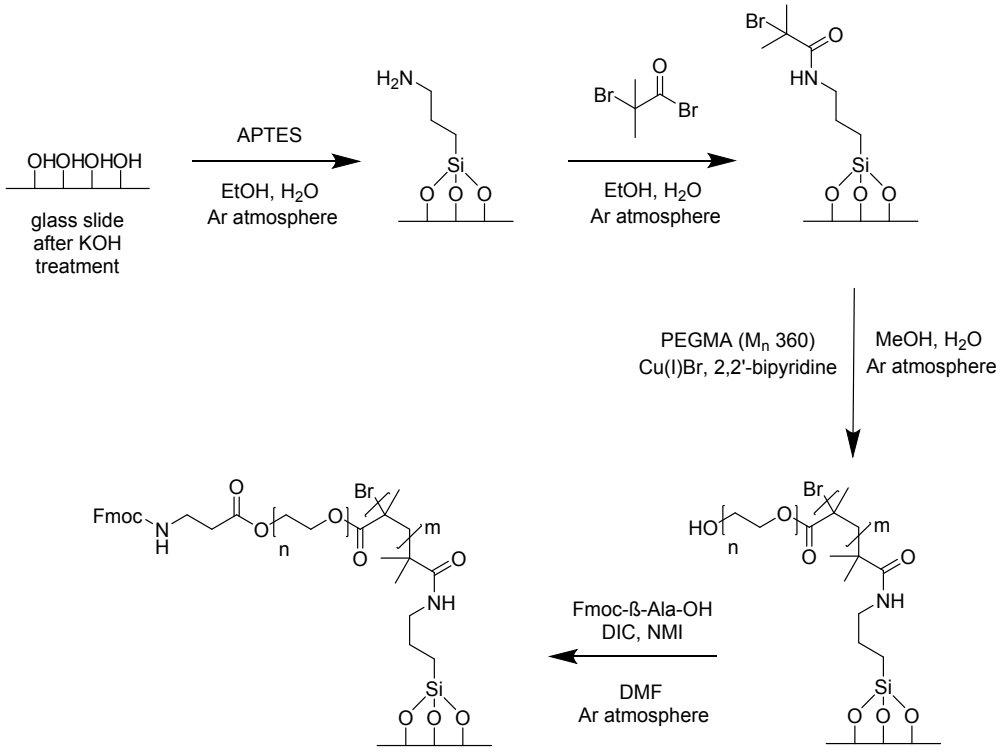
To prepare the donor slide for the laser based deposition: 1.00 mL of a mixture of an Fmoc-Gly-OPfp and resin (S-LEC-P LT 75 52) (10:90 % w/w) in DCM (123 $mgmL^{-1}$) was spin-coated onto a polyimide foil covered microscopy slide with the following parameters: 50 rps for 10 sec followed by 100 rps for 40 sec.

A Fmoc-deprotected PEGMA-co-MMA surface functionalized with three β-Ala's or with β-Ala-β-Ala-β-Ala-Lys(Boc)-Gly-Gly was used to perform an U-4CR with 0.6 M Fmoc-β-Ala-OH, hexanal and cyclohexyl isocyanide according to the above described protocol. After a capping step, the slides were Fmoc-deprotected and used as acceptor slides for laser-induced forward transfer.

A lasing set-up already described in literature was used for the laser-induced forward transfer.[1] A green laser at wavelength 532 nm (FSDL-532-1000T, 1W; Frankfurt Laser Company, Friedrichsdorf/ Germany) was operated at 5 V and with the following laser parameter: relative laser power 100%, pulse duration 0.1 to 10 ms (step size 0.14)

The slide was baked in the oven under argon atmosphere at 90 or 110 °C for 1.5 h to couple the transferred Fmoc-Gly-OPfp to the deprotected Ugi product. After a cooling down period, the slides were flushed thoroughly with DCM and rocked in DCM for 3 × 5 min. Then, the slides were placed in an ultrasonic sound bath for 30 sec followed by 5 min rocking in fresh DCM, the ultrasonic sound bath step followed by rocking in fresh DCM was repeated three times. Finally the slides were dried in a stream of argon. Each coupling step was followed by a capping step. After capping, the surface was Fmoc-deprotected and stained with TAMRA-NHS.

1.2.10. Synthesis of pure poly(ethylene glycol) methacrylate (PEGMA) synthesis surface:



S. Scheme. 3: Schematic synthesis pathway of the pure PEGMA synthesis surface functionalized with one Fmoc-β-Ala as anchor for peptide synthesis.

10 Microcopy slides were gently rocked for 3 h in 250 mL of a 1 M KOH in isopropanol solution, the slides were washed with deionized water 2 × 5 min, sonicated in ultrapure water 5 × 1 min and finally washed with ethanol (EtOH), each washing step with about 250 mL. Afterwards, the slides were immersed in 250 mL of a solution of APTES/ ultrapure water/ EtOH (absolute) (2.9:2.4:94.7 % v/v) under argon atmosphere overnight. Then, the reaction mixture was removed and the slides were immediately flushed thoroughly with EtOH, followed by 5 × 1 min washing steps in ultrasonic sound bath in EtOH, each washings step was conducted with 250 mL solvent. Finally, the slides were baked in the oven for 1 h at 120 °C under argon atmosphere.

The APTES functionalized slides were immersed in 250 mL of a solution of 0.6 mM DIPEA and 0.2 mM α-bromoisobutyryl bromide in DCM (dry) under argon atmosphere overnight. Then, the slides were washed with 250 mL of DCM, 2 × 3 min, followed by 2 × 2 min with MeOH. Finally, the slides were dried under a stream of argon.[2]

The slides were immersed in 250 mL of a solution of poly(ethylene glycol) methyl methacrylate/ ultrapure water/ MeOH (33:33:33 % v/v) containing 50 mM 2,2'-bipyridine and 30 mM copper(I)bromide under argon atmosphere, overnight. Then, the slides were washed, each time with 250 mL of solvent, 1 × 5 min with MeOH/ ultrapure water (50:50 vol-%), 3 × 5 min with ultrapure water and 2 × 3 min with MeOH.[2] Finally, the slides were dried under a stream of argon and they were kept under argon atmosphere at 4 °C until further use. The slides were then functionalized with the first β-Ala according to the procedure described below.

1.2.11. Functionalization with Fmoc-β-Ala-OH

The pure PEGMA slides were functionalized with one Fmoc-β-Ala-OH as an anchor to the synthesis film. PEGMA-co-MMA slides, which were used in the combinatorial laser-induced forward transfer experiments, were functionalized with two additional β-Ala's, before starting the synthesis of the sequence or performing the U-4CR on the surface.

A solution of 0.2 M Fmoc-β-Ala-OH in DMF (dry) and 0.4 M 1-methylimidazole in DMF (dry) with 1.2 eq. of DIC was poured over a Fmoc-deprotected synthesis slide. The slide was kept for at least 2 h under argon atmosphere, when the first Fmoc-β-Ala was coupled to the hydroxyl groups of the PEGMA synthesis film, the reaction was performed overnight. Then the slide was washed 3 × 5 min with DMF and 2 × 3 min with MeOH. Finally, the slide was dried under a stream of argon.[2] Then, the slide was capped overnight. Slides, requiring a spacer, were Fmoc-deprotected and the procedure was repeated until the desired length of β-Ala's for the spacer was reached or it was proceeded with coupling of the next amino acid.

To determine the Fmoc-β-Ala loading of the slides, they were deprotected by pipetting 1 mL of deprotection solution (see Fmoc-deprotection) onto the slide for 20 minutes. Then, the solution was removed by pipet again and the Fmoc-value was determined photo-spectrometrically at 301 nm for 750 µL of the removed solution and the loading was calculated according to literature.[5] Typical values were around 3.5 nmol × cm^{-2}.

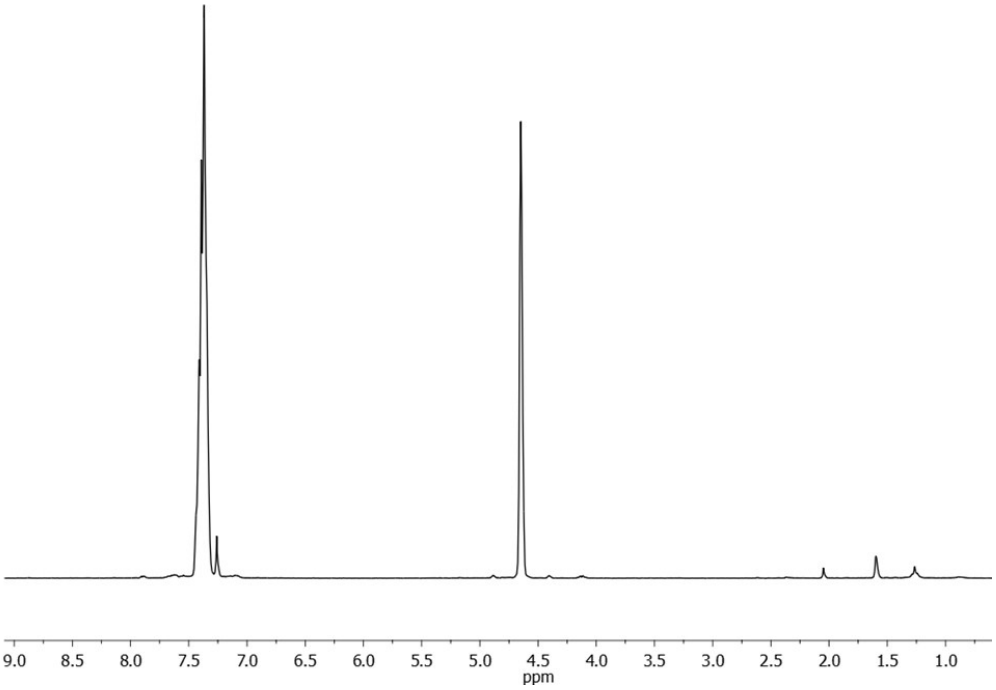
1.2.12. Functionalization of a PEGMA-co-MMA surface with amino acid derivatives from solution:

The Fmoc-deprotected PEGMA-co-MMA synthesis slide, functionalized with a three β-Ala spacer was covered with 1.00 mL of a 0.2 M solution of the Fmoc-protected and OPfp-activated amino acid in DMF (dry) under argon atmosphere at room temperature for 3 to 10 h. Subsequently, the slide was washed with DMF, 3 × 5 min, directly followed by a capping step.[2] Then, the whole procedure was repeated to add the next amino acid.

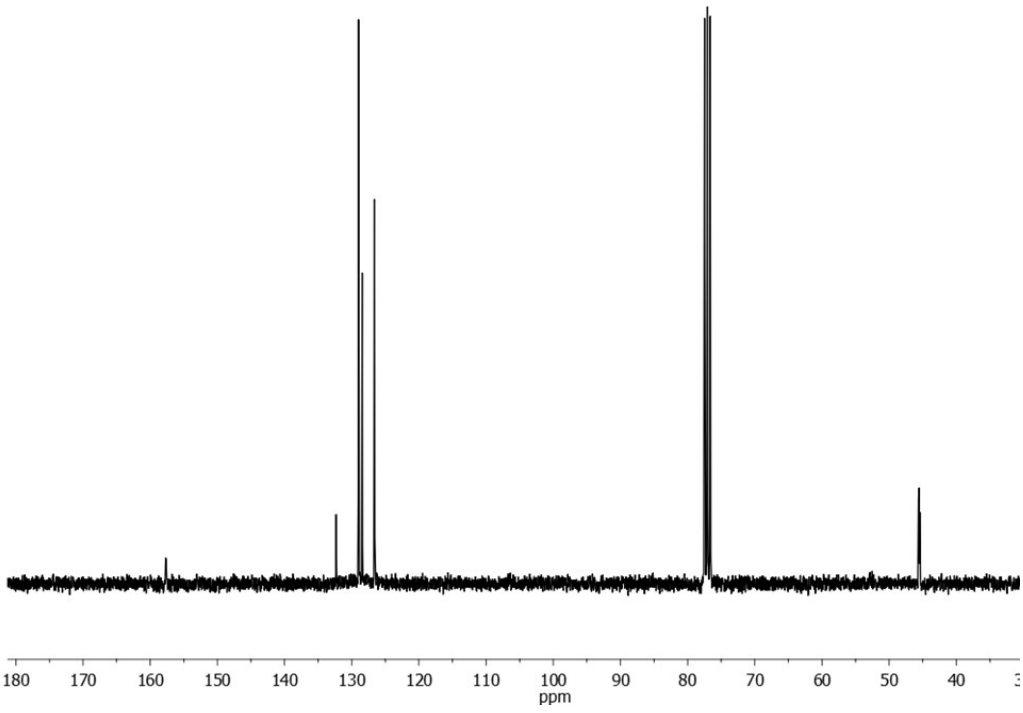
1.2.13. Synthesis of benzyl isocyanide:

Benzyl isocyanide was synthesized according to literature procedure.[6] The amount of chemicals were doubled and in the second step, instead of 0.728 eqv., 2.77 eqv. of TEA were used. Benzyl isocyanide was obtained as a brown liquid (3.22 g, 25%). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.46-7.29 (m, 5H, Ar-*H*), 4.65 (s, 2H, CH_2) ppm. ^{13}C NMR: ($CDCl_3$, 75 MHz): δ 157.69, 132.34, 128.99, 128.43, 126.61, 45.63, 45.53, 45.44 ppm.

^{1}H NMR ($CDCl_3$, 300 MHz)



^{13}C NMR ($CDCl_3$, 75 MHz)



2. Instruments:

2.1. Fluorescence scanners

Analysis of fluorescently labeled synthesis surfaces was performed with an Odyssey Infrared Imager by LICOR Biosciences (Lincoln/ USA). Slides were scanned with a resolution of 21 µm, at intensity 3, 5

or 7 in the 700 nm channel for DyLight 680 staining. The slides used in the laser based transfer were analyzed with a GenePIX 4000B microarray scanner by Molecular Devices (Sunnyvale/ USA). These slides were scanned with a resolution of 5 µm, photo multiplier tube gain 450 and laser intensity 33% in the 532 nm channel. ImageJ or the respective software of the scanner were used to enhance contrast and brightness of the scanned objects after scanning. ImageJ was also used to determine the grey values of the scans from the unmodified images and to make 3D-models of spots.

2.2. Matrix–assisted Laser Desorption/Ionization Time of Flight (MALDI-ToF) measurements

Matrix-assisted Laser Desorption/Ionization Time of Flight (MALDI-ToF) measurements were performed at the DKFZ in Heidelberg on an Ultraflex™ TOFTOF I instrument (Bruker Daltonik, Bremen/ Germany) equipped with a nitrogen laser. The instrument was operated with positive-ion reflecton mode: ion source voltage 1 (ion acceleration voltage) 25.0 kV, ion source voltage 2 (first extraction plate) 21.9 kV, ion source lens voltage 6 kV and reflectron voltage 26.3 kV.

As the matrix, one droplet of 2.5-dihydroxybenzoic acid (Bruker, Daltonik) (20 $mg.mL^{-1}$) was dissolved in acetonitrile/0.1% TFA in water (30:70 vol-%) and placed on a ground steel target. A peptide calibration standard II from Bruker Daltonik was used. The used software was FlexControl version 2.4 for instrument control and FlexAnalysis version 2.4 for spectrum processing.

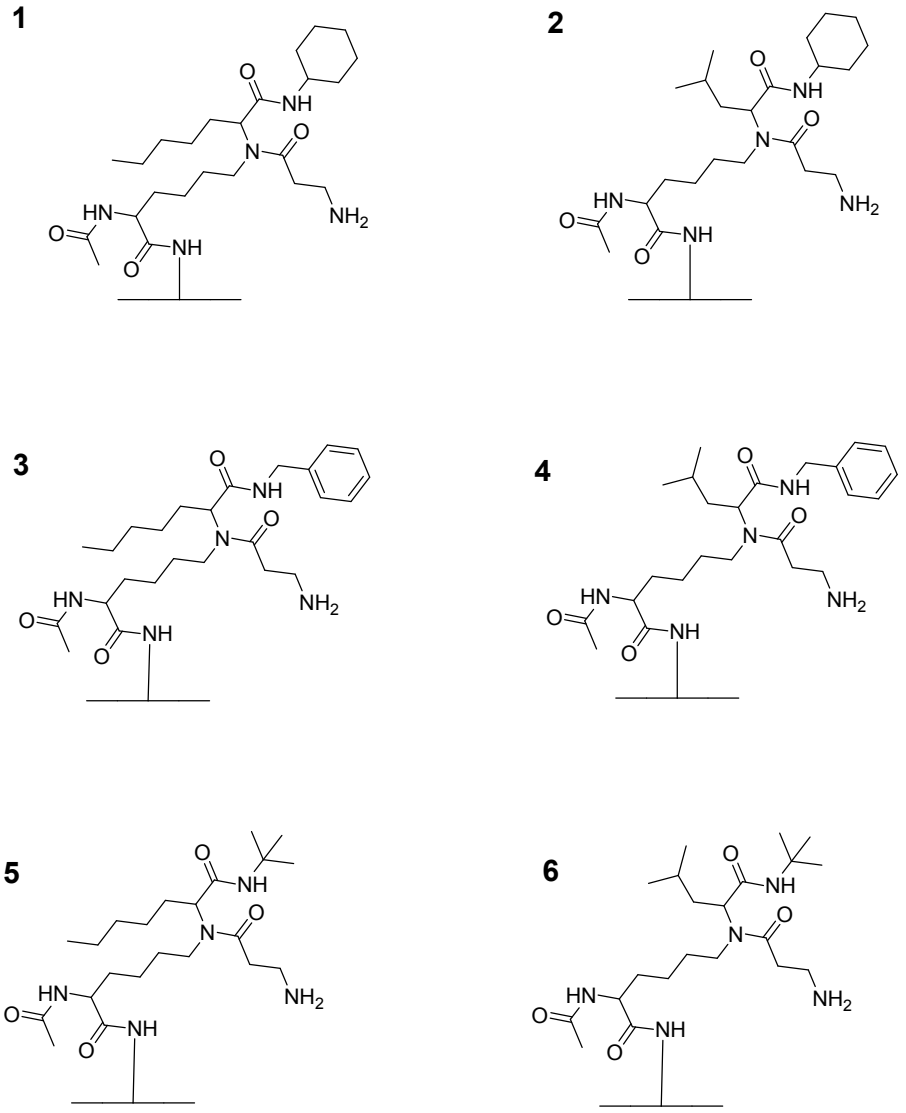
2.3. NMR

NMR spectra were measured on a Bruker AVANCE DPX operating at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR.

3. Supporting data

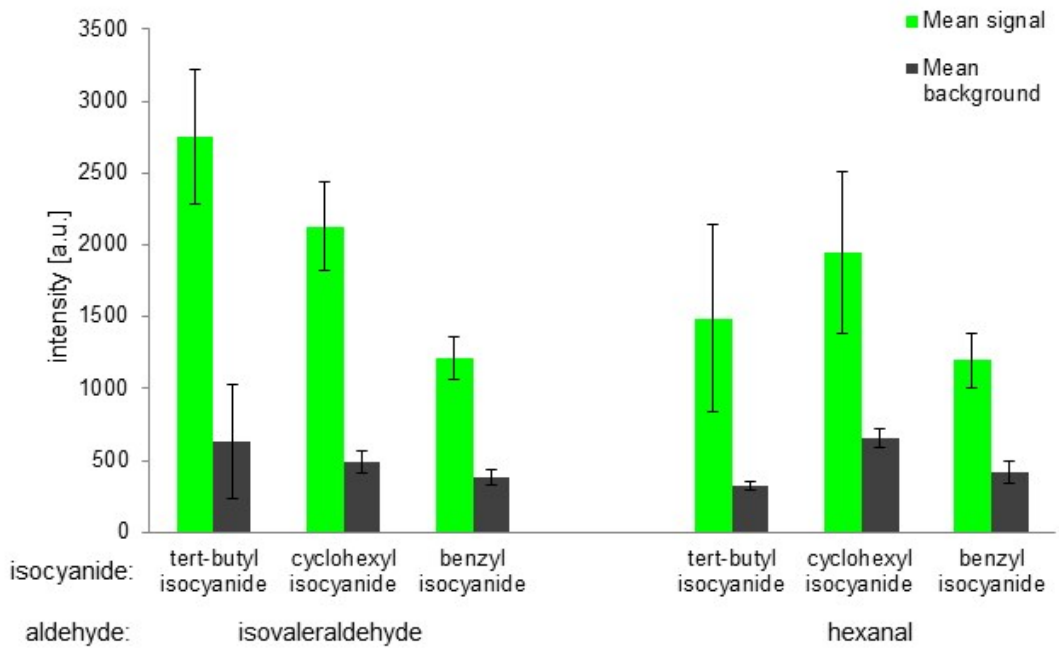
3.1. Analysis of Fluorescence Scans

3.1.1. U-4CR functionalized arrays with alternating Lys and Gly spots



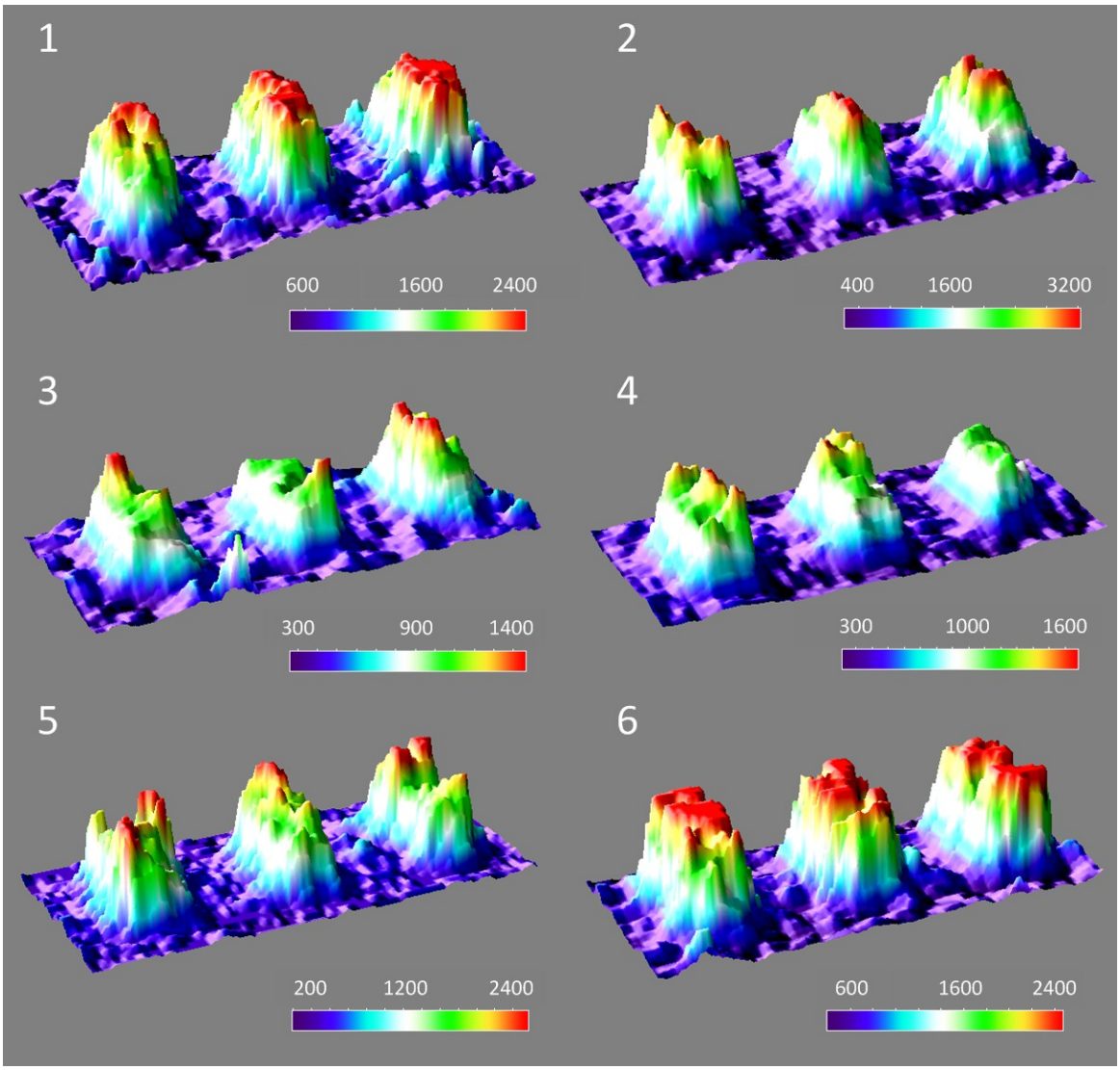
S. Fig. 1: Products of the aldehyde and isocyanide variation experiments, all with the amino component from a side chain deprotected lysine bound to the array and with Fmoc-β-Ala-OH as the carboxylic acid, the aldehyde and the isocyanide component were varied: (**1**) hexanal and cyclohexyl isocyanide, (**2**) isovaleraldehyde and cyclohexyl isocyanide, (**3**) hexanal and benzyl isocyanide, (**4**) isovaleraldehyde and benzyl isocyanide, (**5**) hexanal and *tert*-butyl isocyanide, (**6**) isovaleraldehyde and *tert*-butyl isocyanide.

All arrays were scanned in the Odyssey scanner at a resolution of 21 µm and an intensity of 5 and 7. Separately for both intensities, the grey values of each Lys spot (between 96 and 248 spots as the size of the arrays varied due to the shape of the slide) and a corresponding background spot for each Lys spot were determined and the mean over all measured spots was calculated. Error bars were determined by calculating the standard deviation of the measured grey values. Below the mean values of the fluorescence intensity of the spots and the background belonging to the comparison diagram for intensity 5 are given (see **S. Fig. 2**). For intensity 7, see **Fig. 3**.



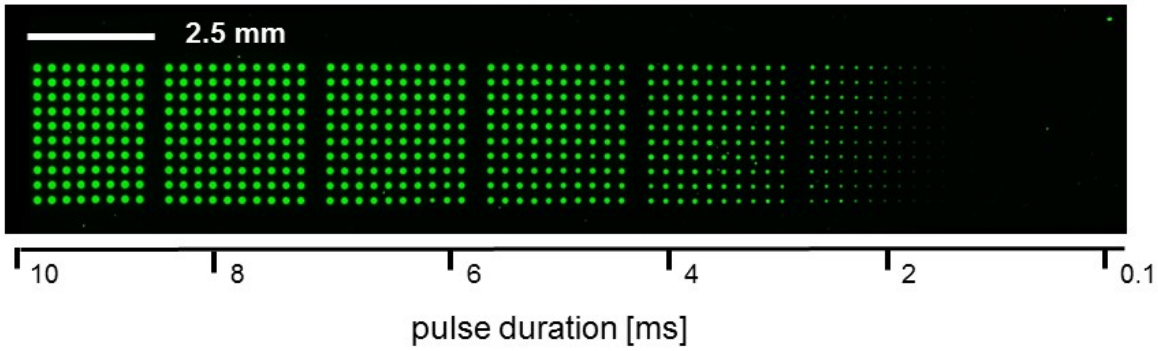
S. Fig. 2: Plotted means of the determined grey values of the spots and the corresponding background spots for the U-4CR products derived of the reaction of Fmoc-β-Ala-OH and different combinations of aldehydes and isocyanides. The amino group was generated by side chain deprotection of Ac-Lys(Boc), which was bound to the PEGMA-co-MMA surface. After capping and Fmoc-deprotection, the arrays were stained with DyLight 680-NHS and scanned in the Odyssey scanner at intensity 5 with a resolution of 21 µm.

The grey values of the fluorescent signal (intensity 5) for three representative spots of each array were also plotted as fluorescence height intensity maps with ImageJ (see **S. Fig. 3**).

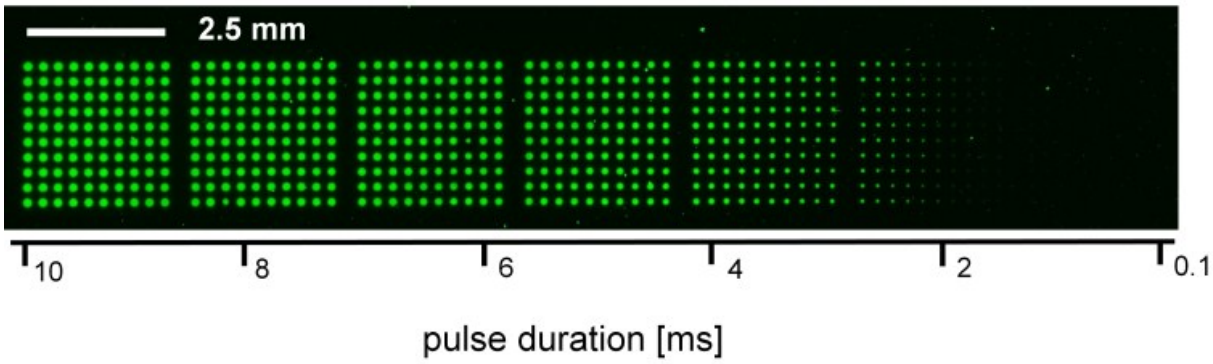


S. Fig. 3: Height-intensity maps of the fluorescent signal for three exemplary spots of the U-4CR products between a side-chain deprotected Ac-Lys, Fmoc-β-Ala-OH and different isocyanides and aldehydes: (1) cyclohexyl isocyanide and hexanal; (2) cyclohexyl isocyanide and isovaleraldehyde; (3) benzyl isocyanide and hexanal; (4) benzyl isocyanide and isovaleraldehyde; (5) *tert*-butyl isocyanide and hexanal; (6) *tert*-butyl isocyanide and isovaleraldehyde. All were stained with DyLight 680-NHS after an Fmoc-deprotection step and scanned at intensity 5. The raw grey values obtained from the scan were used as base for the 3D-models.

3.1.2. U-4CR product functionalized with Fmoc-Gly-OPfp by combinatorial laser-induced forward transfer



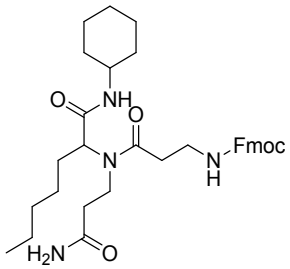
S. Fig. 4: Fluorescence scan of spots at laser parameter variation. Fmoc-Gly-OPfp was transferred to a Fmoc-deprotected PEGMA-co-MMA slide functionalized with a three β-Ala spacer further functionalized with an U-4CR product between hexanal, cyclohexyl isocyanide and Fmoc-β-Ala. The coupling was performed at 110°C for 1.5 hours. After capping and an Fmoc-deprotection, the *N*-terminus was stained with TAMRA-NHS. The slide was scanned in the GenePix Scanner. Contrast and brightness were adjusted.



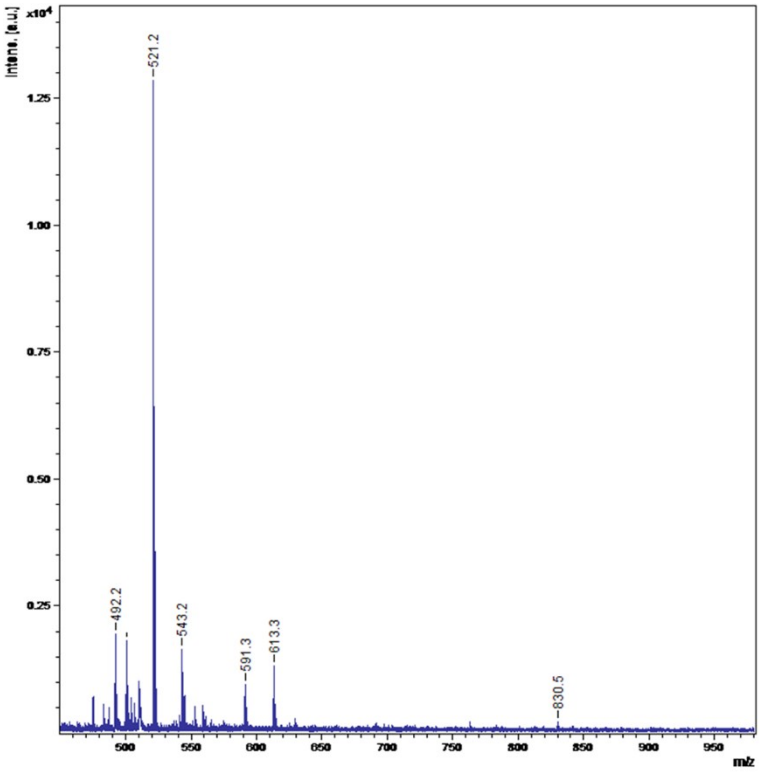
S. Fig. 5: Fluorescence scan of spots at laser parameter variation. To an Fmoc-deprotected PEGMA-co-MMA slide functionalized with β-Ala-β-Ala-β-Ala-Lys(Boc)-Gly-Gly-U-4CR (hexanal, cyclohexyl isocyanide and Fmoc-β-Ala), Fmoc-Gly-OPfp was transferred and coupled at 90°C. After capping and an Fmoc-deprotection step, the *N*-terminus was incubated with TAMRA-NHS. The slide was scanned in the GenePix Scanner. Contrast and brightness were adjusted.

3.2. MALDI-ToF measurements of detached U-4CR products (for detachment procedure, see 1.2.7)

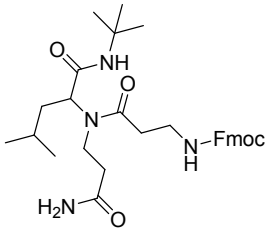
3.2.1. Hexanal and cyclohexyl isocyanide



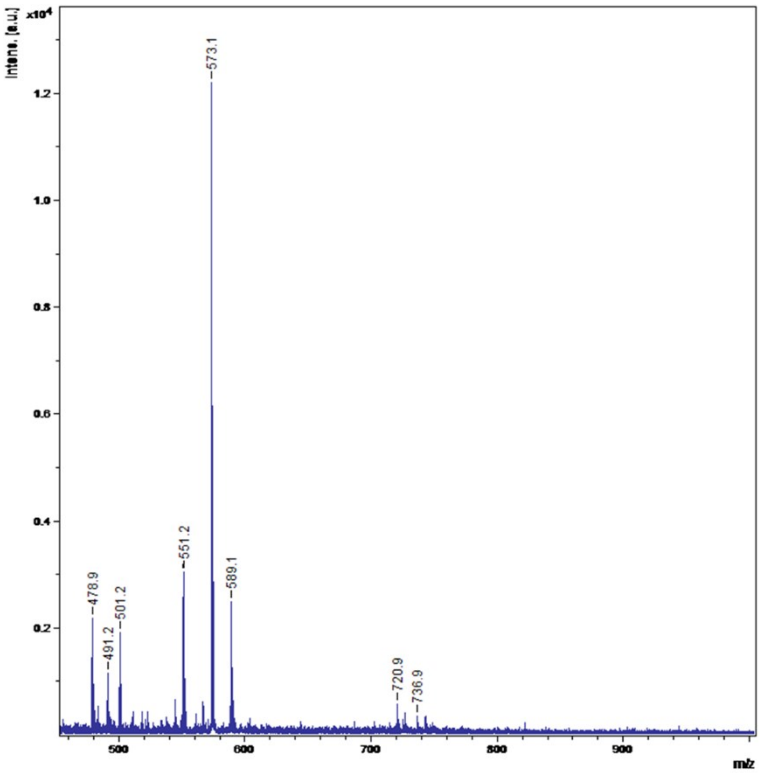
MS (MALDI): m/z cal. 590.8, measured 591.3[(M+H)]$^+$; 613.3 [(M+Na)]$^+$.



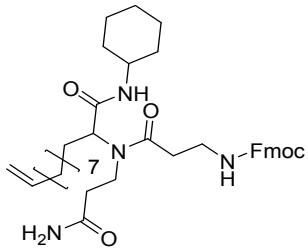
3.2.2. Isovaleraldehyde and *tert*-butyl isocyanide



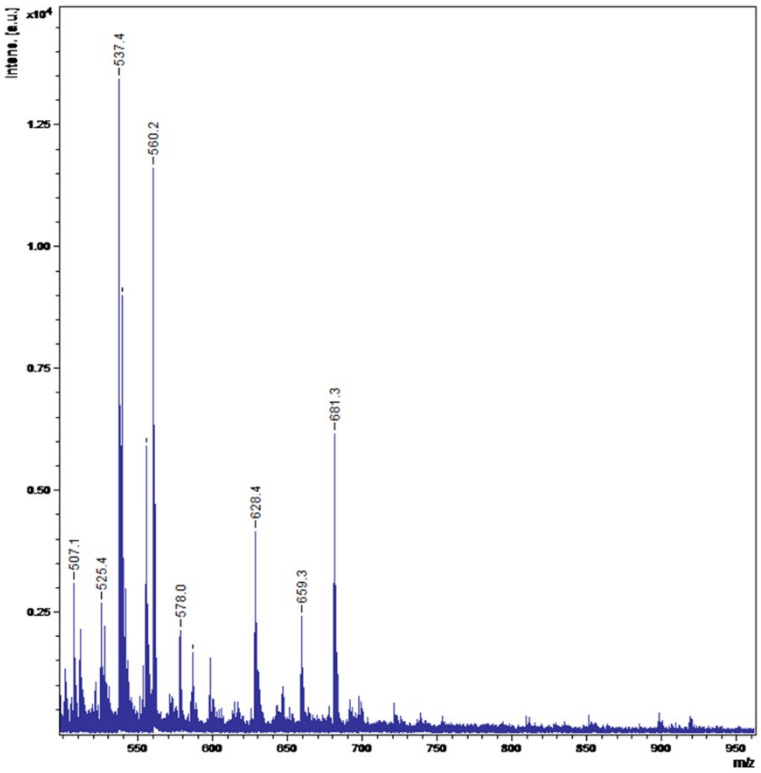
MS (MALDI): m/z cal. 550.7, measured 551.2 $[(M+H)]^+$; 573.1 $[(M+Na)]^+$; 589.1$[(M+K)]^+$.



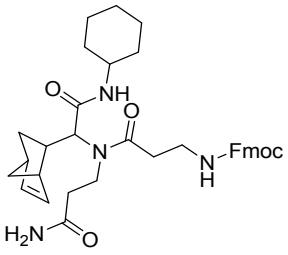
3.2.3. Undecenal and cyclohexyl isocyanide



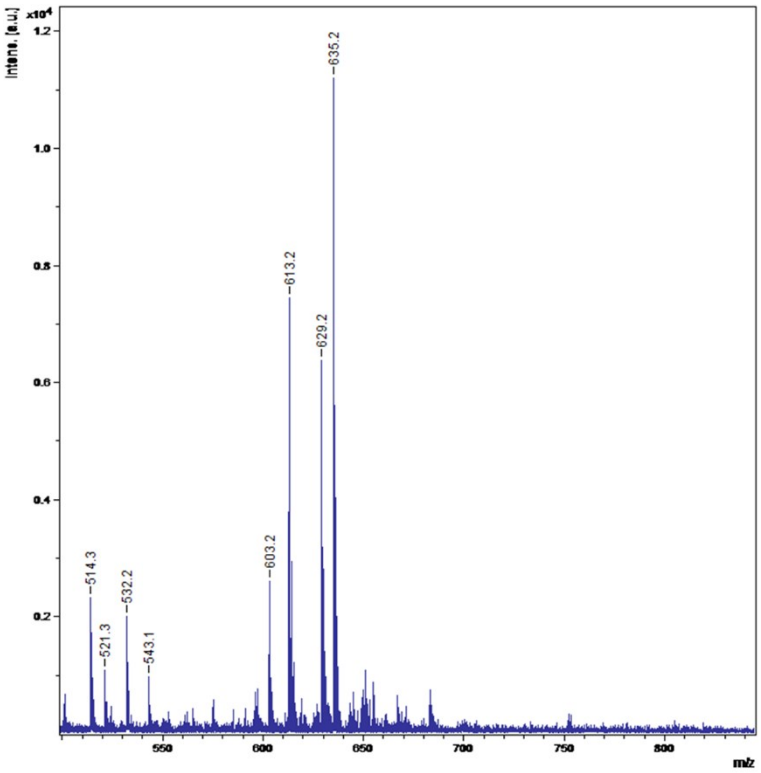
MS (MALDI): m/z cal. 658.9, measured 659.3 $[(M+H)]^+$; 681.3 $[(M+Na)]^+$.



3.2.4. Bicyclo[2.2.1] hept-5-ene-2-carboxaldehyde and cyclohexyl isocyanide



MS (MALDI): m/z cal. 612.8, measured 613.2 $[(M+H)]^+$; 635.2 $[(M+Na)]^+$.



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