Complex surface concentration gradients by stencilled ”Electro click chemistry”

Thomas S. Hansen\textsuperscript{1,†}, Johan U. Lind\textsuperscript{1 †}, Anders E. Daugaard\textsuperscript{2}, Søren Hvilsted\textsuperscript{2}, Thomas L. Andresen\textsuperscript{1}, and Niels B. Larsen\textsuperscript{1,*}

Supporting Information

Synthesis of alkyne-PEG-GRGDS and alkyne-PEG-GRDGS (see Figure S1):

\textit{Pentyne-PEG\textsubscript{11}-Gly\textsubscript{5}-(4-iodo-Phe)-Gly-Arg-Gly-Asp-Ser-NH\textsubscript{2} (alkyne-PEG-GRGDS)} was made using solid phase synthesis. Base labile Fmoc was used for protecting groups for the amines, and the employed resin was acid labile rink-amide. 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium-hexafluorophosphate-Methanaminium (HATU), Fmoc-Ser(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-4-Iodo-Phe-OH, Fmoc-Gly-OH and Rink Amide-AM Resin were bought from GL Biochem, Shanghai, China. Fmoc-NH-PEG\textsubscript{11}-COOH, \textit{PEG1080}, was acquired from IRIS biotech, Marktredwitz, Germany. 4-Pentynoic acid, (EDC), 2,4,6-Collidine, tri-fluoro-acetic-acid (TFA), Tri-isopropyl silane (TIPS), ninhydrin, piperidine, and all solvents, were acquired from Sigma-Aldrich.

Before reaction, the resin was swelled in dichloromethane (DCM) for 1½hr. In each coupling step four equivalents of the new amino acid was used. The amino acid was first mixed with 3.9 equivalent (eq.)
HATU coupling reagent and 8 eq. 2,4,6-collidine in dimethylformamide (DMF), then added to the peptide, and allowed to react for 30min. The beads were washed 4 times in DMF and 2 times in DCM, each washing step lasting 30sec. De-protection of the Fmoc group was done by washing twice for 2 min with a 20% solution of piperidine in DMF.

Kaiser tests were performed to test the presence of free amino groups, between each reaction/de-protection step. The coupling of the PEG segment was done by performing two consecutive 1 hr coupling reactions to ensure a successful binding of the PEG segment. The following pentynoic acid step was also allowed to react for 1 hr.

The peptide was cleaved from the resin using a solution of 2.5% vol TIPS, 2.5% vol milli-Q water and 95% TFA. A cleaving time of 3 hrs was used. The cleaving solution containing the peptides was then dried on a rotary evaporator. The material remaining was then gently decanted five times using 5ml diethyl ether.

The product was purified using preparative reverse phase High Performance Liquid Chromatography (RP-HPLC). The RP-HPLC was conducted on a 250mm x 20mm C18 column with a bead size of 5um and a pore size of 100Å. Solvent A: 95/5 % H2O/MeOH 0.1% TFA. Solvent B: 99.9% MeOH/0.1% TFA. The solvents were degassed using a glass filter, prior to their use on the RP-HPLC.

Solutions of the peptides were made in 50% Solvent A/Solvent B. In the RP-HPLC procedure, a linear gradient from 52% to 54% of Solvent A over 16mins were used. UV detection was set to 206 nm (carbonyl) and 257 nm (phenylalanine).

An equivalent procedure was used for the fabrication and purification of the scrambled sequence peptide, Pentyne-PEG$_{11}$-Gly$_{5}$-(4-iodo-Phe)-Gly-Arg-Asp-Gly-Ser-NH$_{2}$ (alkyne-PEG-GRDGS).

Yields after RP-HPLC purification and freeze drying were:
Alkyne-PEG-GRGDS: 41.3mg (16%)

Alkyne-PEG-GRDGS: 48.8mg (19%)

The purity of the products was confirmed by MALDI-TOF MS using a Bruker Reflex IV MALDI-TOF, matrix 50mg/ml 2,5-dihydroxy benzoic acid (DHB) in ethanol, and 0.1-1mg/ml solutions.

Figure S1: The structure of the alkyne-PEG-GRGDS (similar for “scrambled” alkyne-PEG-GRDGS, except for exchange of the last glycine and aspartate moieties).

Synthesis of NTA-alkyne (Prop-2-ynyl 5-(N,N-bis(carboxymethyl)amino)-5-(S)carboxypentanecarbamate) (see Figure S2):

\[ \text{N,N-Bis(carboxymethyl)-L-lysine hydrate (NTA, 0.100 g, 0.4 mmol) and NaHCO}_3 (0.128 g, 1.5 \text{ mmol}) } \] was dissolved in H\(_2\)O (3.5 mL). Propargylchloroformate (0.05 g, 0.4 mmol) in toluene (2 mL) was added slowly at 0 °C. The reaction mixture was stirred overnight at room temperature. Toluene was removed in vacuo and the residue was acidified with DOWEX. The product was isolated after lyophilization as a white solid (79.0 %, T\(_m\) = 135 °C).
IR (cm⁻¹): 3405 (N-H); 3280 (C≡C-H); 3100-2800 (C-H); 2120 (C≡C); 1705 (COOH); 1615 OC(O)N); 1395 (C-N); 1255, 1135 (C-O).

¹H-NMR (300 MHz) D₂O, δH (ppm): 1.2-1.7 (m, 6 H, CH₂-CH₂); 2.92 (t, ⁴J = 2.2 Hz, 1 H, C≡C-H); 3.18 (t, ³J =5.9 Hz, 2 H, OC(O)NH-CH₂); 3.82 (m, 5 H, N-CH₂-COOH, N-CH-COOH); 4.69 (m, 2 H, OCH₂-C≡CH).

¹³C-NMR (75 MHz) D₂O, δC (ppm): 23.6, 26.5, 28.6 (3 C, CH₂-CH₂); 40.1 (1 C, OC(O)N-CH₂); 52.7 (1 C, C≡C-CH₂); 55.5 (2 C, N-CH₂-COO); 68.3 (1 C, N-CH-COOH); 75.7 (1 C, HC≡C); 78.8 (1 C, HC≡C-CH₂); 157.6 (1 C, OC(O)N); 170.5 (2 C, CH₂-COOH); 172.8 (1 C, CH-COOH).

MS (m/z, ESI): 367 [M+Na]+.

Figure S2: Structure of the NTA-alkyne

Synthesis of PEG-alkyne (see Figure S3):

α-Methoxy-poly(ethylene glycol)-ω-pent-4-ynamide, was made by coupling 2.0173g MeO-PEG-NH₂ (PEG1155, 750Da, PDI 1.2, Iris biotech, Marktredwitz, Germany) and 237.3mg (0.9 eq.) 4-Pentyanoic acid (Sigma-Aldrich ) in 60mL dichloromethane using 155.24mg (1.eq.) N-(3-Dimethylaminopropyl)-N´-ethyl-carbodiimide (EDC) (Sigma-Aldrich) overnight.
The reaction mixture was concentrated to dryness using a rotary evaporator and the product was hereafter purified by flash chromatography packed with silica gel (eluent: methanol/DCM 5:95). The product fractions were concentrated on a rotary evaporator and dried overnight on an oil pump. The yield was 916mg (45.6%) output.

MALDI-TOF MS confirmed the successful coupling had taken place. A Bruker Reflex IV MALDI-TOF, matrix 50mg/ml 2,5-dihydroxy benzoic acid (DHB) in ethanol, and 0.1-1mg/ml solutions of the product were applied. TLC (eluent: methanol/DCM 5:95) indicated no presence of the starting materials. This was confirmed by $^1$H- and $^{13}$C-NMR.

$^{13}$C-NMR (63 MHz): ($\delta_C$, ppm) 170.9(N-C=O), 83.08(C≡C-H) 71.9(CH$_3$-O-CH$_2$) 70.6-69.7(O-C-C), 69.2(C-≡C), 58.9(CH$_3$-O-CH$_2$) 39.3(C-≡N=O), 35.2(O=C-C-C) 14.8(C=C=C=C)

$^1$H-NMR (250 MHz): ($\delta_H$, ppm) 6.35(s, H$_N$-C=O), 3.4-3.7(m, O-CH$_2$-CH$_2$-O), 3.35(s, O-CH$_3$) 2.4-2.6(m, O=C-CH$_2$-CH$_2$-C≡C), 1.95(H=C≡C),

Figure S3: Structure of PEG-alkyne
Polymerization of thin films of PEDOT-N₃ onto COC supports:

PEDOT-N₃ electrodes were prepared by in situ polymerization of EDOT-N₃ on injection molded COC (TOPAS 5013, TOPAS Advanced Polymers, Frankfurt, Germany) discs. The discs were cleaned with acetone, IPA, ethanol and water. EDOT-N₃ (see Figure 1A in main paper) (20 mg, 0.15 mmol), Baytron C (0.48 mL, ~40 wt % Fe(III)Tosylate in butanol), and butanol (0.48 mL) were mixed and spin-coated on the COC-discs (20 s at 700 rpm). The samples were placed on a hot plate at 65 °C for 5 min and subsequently washed with water and blown dry in a nitrogen flow, yielding films with a thickness of approximately 150 nm.

Potentiostat and Microscope

The fabrication of the gradient was performed using a VMP multichannel potentiostat (Biologic Science Instruments, Claix, France). The fluorescence of the eGFP treated areas were measured using a Zeiss LSM 5 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) using exciting light at 488 nm and collecting emitted light of wavelength longer than 505 nm.

Nuclear magnetic resonance

NMR spectroscopy of the NTA-alkyne was performed on a 300 MHz Cryomagnet from Spectrospin & Bruker (¹H-NMR at 300MHz, ¹³C-NMR at 75MHz), while the PEG-alkyne was analyzed on a Bruker Avance 250MHz spectrometer (¹H-NMR at 250MHz, ¹³C-NMR at 63MHz). All analyses were performed at room temperature.
Infrared spectroscopy (IR)

IR was performed on a PerkinElmer Spectrum One model 2000 Fourier transform infrared system with a universal attenuated total reflection sampling accessory on a ZnSe/diamond composite.

Fabrication of mask for complex NTA gradients:

A 4 inch silicon wafer was coated by 500 nm copper using electron beam evaporation, before spin coating with COC mr-I T85 (Micro Resist Technology, Berlin, Germany) at 3000 rpm for 30 seconds to yield a 4.5 µm thick layer, as measured with an Ambios XP-2 (Ambios Technology, Inc., Santa Cruz, US) profilometer using a stylus force of 1 mg. After baking at 80°C for 10 minutes a layer of ma-P 1275 (Micro Resist Technology, Berlin, Germany) was spin coated on top at 3000 rpm for 30 seconds, and the sample was rebaked at 80°C for 10 minutes. The photoresist was exposed through a chrome mask in a Karl Süss MA4 mask aligner (Munich, Germany) with a dose of 400 mJ/cm². The chrome mask motif consisted of a square grid of 100 µm diameter holes with a center-to-center distance of 200 µm in both lateral dimensions (see Fig. 5A of the main text for a schematic). The photoresist was developed using ma D 331 (Micro Resist Technology, Berlin, Germany) for 60 seconds. The wafer was then etched in a Reactive Ion Etcher (Plasmatherm 740, Unaxis, St Petersburg, FL) for 15 minutes at 300 W and 7 Pa O₂. Finally the remaining photoresist was removed by washing in acetone, isopropanol, ethanol, and MilliQ water.
The gradient fabrication setup:

**Figure S4**: Top: The PEDOT-N$_3$ coated TOPAS disc (left) and the copper plate with transfer adhesive (right). A slit has been cut in the transfer adhesive using a CO$_2$ laser. The rim is also made of transfer adhesive and is used for defining the spacing between the PEDOT-N$_3$ surface and the copper electrode. Bottom: the assembled setup without clamps and connectors. The TOPAS disc has two holes for inlet and outlet of the alkyne/CuSO$_4$ solution.
Figure S5: XPS linescan data on gradients made using a concentration of 1 mM EBTB, a potential of -0.5V, a reaction time of 5 minutes and a spacing of 190 µm. A test sample was made where the solution contained 9 mM of the inert NiSO$_4$ to test the influence on electrolyte conductivity on the gradient profile. The profile of the 1 mM CuSO$_4$ + 9 mM NiSO$_4$ compared to 10 mM CuSO$_4$ indicates that the shape of the 10 mM CuSO$_4$ is most likely linked to conductivity of the electrolyte.
Figure S6: XPS linescan data on gradients made using a concentration of 1 mM EBTB, 1 mM CuSO₄, a reaction time of 5 minutes, a spacing of 190 μm and various potentials. The figure shows that only little effect is gained by increasing the potential from -0.5V to -1.5V. The control with 0.0V shows that no chemical modification is observed when no potential is applied.
COMSOL simulation

The setup was simulated using COMSOL Multiphysics 3.5 (COMSOL AB, Stockholm, Sweden) using the packages “Conductive media DC” and “Diffusion”.

**Scheme S1:** The Values used for the COMSOL simulation. The diffusion coefficients and the molar conductivity of CuSO₄ in DMSO were assessed from similar values in water. The reaction rate constant was determined from figure S9.

<table>
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<th>Variable</th>
<th>Symbol</th>
<th>Estimated value/Units</th>
<th>Dimensionless variable</th>
<th>Dimensionless values used in the simulations</th>
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<td>Spacing Height</td>
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<tr>
<td>Length</td>
<td>L</td>
<td>m</td>
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<tr>
<td>Film thickness</td>
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<td>( \hat{d} = \frac{d}{h} )</td>
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<td>Slit width</td>
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<td>Applied potential</td>
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<td>( \hat{V} = \frac{V}{V_0} = 1 )</td>
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</tr>
<tr>
<td>Time</td>
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<td>Concentration of alkyne</td>
<td>( c_{\text{alk}} )</td>
<td>1 mol/m³</td>
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<td>Concentration of azide</td>
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<td>Concentration of Cu(II)</td>
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<td>Concentration of Triazole</td>
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<td>mol/m³</td>
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<tr>
<td>Diffusion coefficients</td>
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<tr>
<td>Current</td>
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<td>A</td>
<td>( \hat{I} = \frac{I}{h} )</td>
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<tr>
<td>Reaction Rate Constant</td>
<td>k</td>
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<td>( \hat{k} = \frac{k}{k_{\text{alk,0}}} )</td>
<td>( 10^4 )</td>
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**Figure S7:** The geometry used in the COMSOL simulation. The azides and triazoles are bound to a thin area in the bottom resembling the PEDOT-N$_3$ layer. The symmetry plane makes it possible to simulate only one half of the geometry.
**Figure S8:** Example of the dimensionless output from a COMSOL simulation using the values in scheme S1. A) is the electric potential, B) is the alkyne concentration, showing signs of depletion in the volume below and next to the stencil opening, C) is the Cu(I) concentration, D) and E) are the Triazole and Azide concentrations, respectively, bound in the PEDOT-N$_3$-mimicking subdomain.
Figure S9: COMSOL simulation of the resulting triazole surface concentration for a range of values of the dimensionless reaction rate $\tilde{k}$ compared to experimental data produced at -0.5V, 1 mM CuSO$_4$, 1 mM EBTB, electrode spacing 190 µm and 5 minutes reaction time. The optimal value of the reaction rate constant is found to be $\tilde{k}=10000$ for the current setup.
Figure S10: Numerical simulation of the triazole concentration as a function of distance for varying values of $c_{\text{CuSO}_4}$ compared to experimental data using an applied potential of -0.5V, 10 mM CuSO$_4$, 1mM EBTB, electrode spacing 190 µm, and 5 minutes reaction time. The shoulder observed in the experimental data at large CuSO$_4$ values can partly be reproduced using the numerical simulations. The simulations reveal that the distinct shape with the shoulder is caused by depletion of the alkyne.