Supporting Information

Monitoring Clinical Levels of Heparin in Human Blood Samples with an Indicator-Displacement Assay

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GENERAL

All commercial chemicals were used as received without further purification. DGLs were provided by the COLCOM company (G4: batch #1207-04, G3: batch #1204-03). Poly-L-lysine hydrochloride (PLL_{400}, Mw=66 kDa) was purchased from Alamanda, and GAGs from Sigma-Aldrich: Heparin sodium salt from porcine intestinal mucosa (207 USP/mg, ref: H3399-50KU), Chondroitin sulfate A from sodium bovine trachea (ref: C9819-5G), Hyaluronic acid sodium salt from streptococcus equi (ref: 94137-10MG). Sheep serum was purchased from Sigma-Aldrich (ref: S2263-100ML), while human blood samples – collected in sodium citrate tubes – were provided by the Etablissement Français du sang (EFS). As serum and blood are viscous liquids, low-binding tips for automatic pipettes were used.

All solutions were prepared in 10 mM HEPES buffer, pH 7.8. The buffer was prepared using MilliQ deionized water (18 MΩ). HPLC purifications were performed using an Armen SPOT Prep system, on a Reveleris flash cartridge (reversed phase C18, 40 µM particles, 40 g) operated at 9 $mL.min^{-1}$. HPLC analyses were performed on a Waters equipment including a Waters 996 photodiode array detector and a Waters 2690 separation module. The analytical column (Thermo Scientific 3 µm C18 particles, 50 x 2.1 mm) was flooded at 0.2 $mL.min^{-1}$. For the two previous HPLC setups, a gradient from 90:10 H_2O (0.1 % TFA)/MeCN (0.1 % TFA) to 10:90 H_2O (0.1 % TFA)/MeCN (0.1 % TFA) over 30 minutes was operated, with UV/Vis monitoring at 215 and 437 nm. ESI-MS spectra were recorded on a Synapt G2 mass spectrometer. Fluorescence measurements were performed on a Berthold Tristar LB 941 microplate reader (λ_{ex} = 485 nm and λ_{em} = = 535 nm).

All experiments were performed at least in duplicate.

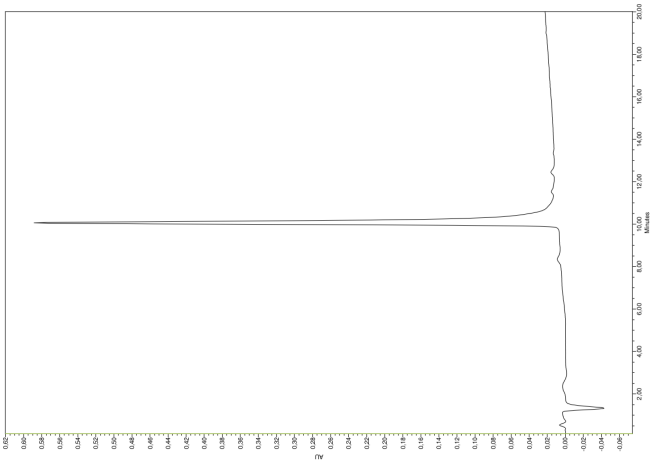
SYNTHESIS

Assembly of the linear protected peptide Fmoc-Asp(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp-(OtBu)-ChloroTrityl resin was carried out manually with a traditional Fmoc/tBu strategy in a glass reactor with a glass frit. 250 mg of Chlorotrytil resin (1.3 mmol/g loading) were made to swell in 5 mL of dichloromethane (DCM). Fmoc-Asp(OtBu)-OH (74 mg, 0.18 mmol) was added, followed by DIEA (0.1 mL, 0.6 mmol). The suspension obtained was stirred for 90 min at room temperature. The supernatant was removed by

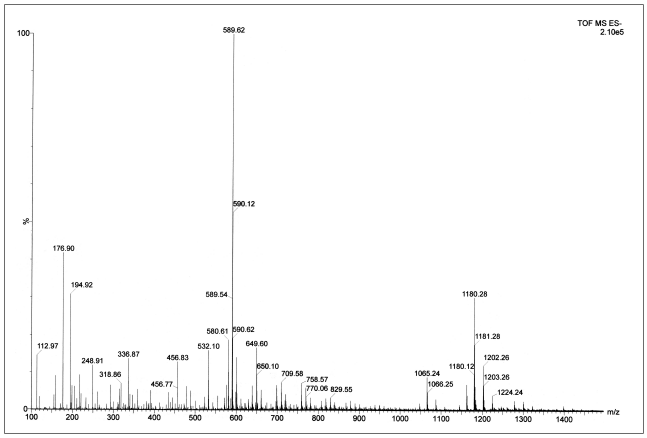
suction and then the resin was capped with DCM/MeOH/DIEA (17:2:1, 5 mL, 1 h), then DCM/MeOH/DIEA (17:2:1, 5 mL, 10 min). Fmoc deprotection reactions were performed with DMF/piperidine (8:2, 3x3 mL, 5 min each) and the resin was washed with DMF (5x3 mL, 1 min each). A suspension of Fmoc-Asp(OtBu)-OH (123 mg, 0.3 mg), PyBop (156 mg, 0.3 mg) and DIEA (0.1 mL, 0.6 mmol) in 5 mL of DMF was stirred for 45 min. Each coupling reaction was performed in duplicate in order to avoid the risk of deletion. The final introduction of the 5(6)-carboxyfluorescein (CF) label was performed as follow, reducing lighting exposure as much as possible:[1] 50 mg of H_2N-Aps(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp(OtBu)-Asp-(OtBu)-ChloroTrityl™ were made to swell in 5 mL of DMF. CF (113 mg, 0.3 mmol), DIC (0.05 mL, 0.3 mmol) and HOBt (46 mg, 0.3 mmol) were added and the suspension was stirred for 1 h. The fully deprotected and labeled peptide was recovered directly upon acid cleavage with TFA/TIPS/H_2O (95:2.5:2.5, 4 mL, 1 h), followed by TFA/TIPS/H_2O (95:2.5:2.5, 2x3 mL, 1 min each). The combined filtrates were concentrated under reduced pressure. Crude D7CF was obtained by precipitation with Et_2O.

Analyses and purification of the synthesized D7CF were performed by HPLC column, as previously mentioned (see GENERAL section). Retention time = 11.06 minutes. Yield = 49%.

HPLC trace:



MS spectrum:



m/z:

1224.24 → $[M+2Na-3H]^-$

1202.26 → $[M+Na-2H]^-$

1180.28 → $[M-H]^-$

1065.24 → $[M-Asp-H]^-$

589.62 → $[M-2H]^{2-}$

METHODS

Typical isotherm experiment

Eppendorf tubes were filled with 100 µL of a stock solution of G4 (final concentration: 195 nM) and variable volumes of a D7CF solution (final concentrations: from 1 to 14 µM). The volume of each sample was completed to 2 mL with buffer. The samples were shaken for 2 hours at room temperature.

Then, 500 µL of the previous solutions were centrifuged at 3500 g (Sigma 302-K, 8000 rpm) for 1 min over an inert membrane (Sartorius Vivacon® 500, Molecular Weight Cut-Off: 10 kDa). The process allowed 20 µL of solutions to cross the membranes, which were transferred into 96 wells plates. The volume of each well was completed to 150 µL with buffer, and the fluorescence intensity was measured.

The same procedure (samples containing only D7CF) was repeated for calibration.

Typical Heparin titration in human blood

Eppendorf tubes were filled with 100 µL of pure human blood. Variable volumes of a stock solution of heparin in 10 mL HEPES buffer (pH 7.8) were added and the final volumes were adjusted to 200 µL with buffer. Heparinized blood samples were allowed to stir for 1 h at room temperature before use.

In a typical titration experiment, microplate wells were filled with 95 µL of buffer, 20 µL of a G4 solution in buffer (final concentration in the well: 55 nM), 20 µL of D7CF solution in buffer (final concentration in the well: 1.52 µM), and finally 15 µL of the previous heparinized blood samples. The fluorescence intensity was then followed for 2 hours, with measurements every 2 min.

Fitting the titration data

Binding isotherm

First, the mean number of ligands bounded to one molecule of receptor n was determined from the following equation:

$$n = \frac{[L]_{tot} - [L]}{[S]_{tot}}$$

, where $[L]_{tot}$ is the total concentration of ligand, [L] is the free ligand concentration, and $[S]_{tot}$ the receptor concentration. Then, n was plotted over [L] to graphically determine n_{max}, the maximum number of ligands per molecule of receptor.

Scatchard plot

n/[L] was plotted versus n. As the curve obtained was not linear, 2 binding models were proposed:

- **two non-equivalent independent binding sites**

The following equation

$$n=\frac{n_1 k_1[L]}{1+k_1[L]}+\frac{n_2 k_2[L]}{1+k_2[L]}$$

was used to fit the experimental points from the isotherm experiments. n_1 is the number of ligands for the first binding site, and k_1 the association constant for this site. Fitting with Origin gave the following results:

$n_1 = 41.2 \pm 2.9$

$k_1 = 3.56\text{x}10^8 \pm 7.9\text{x}10^7\ M^{-1}$

$n_2 = 38.6 \pm 5.0$

$k_2 = 3.02\text{x}10^6 \pm 1.5\text{x}10^6\ M^{-1}$

The confidence intervals are the standard deviation of the fitted parameters. As it is well know that the first portion of the Scatchard plot can be fitted with a linear equation[3]:

$$y=-k_1 n+n_1 k_1$$

, the second portion cannot be fitted with the parameters previously obtained. A simple linear regression was applied to this portion, to give the following coefficients:

slope = -0.0174

intercept = 1.25

- **multiple equivalent non-independent binding sites**

The Hill equation can be written as:[4]

$$\theta=\frac{1}{\left(\frac{K_D}{[L]}\right)^z+1}$$

, where K_D is the dissociation constant of the complex, z the Hill coefficient, and θ the fraction of occupied sites on the receptor. n as been previously defined as the number of occupied sites on the receptor. Therefore:

$$\theta=\frac{n}{n_{max}} \quad \text{, and} \quad \frac{n}{n_{max}}=\frac{1}{\left(\frac{K_D}{[L]}\right)^z+1}$$

,which is equivalent to:

$$\frac{n_{max}-n}{n}=\left(\frac{K_D}{[L]}\right)^z$$

Taking the logarithm of the previous equation finally gives:

$$\log\left(\frac{n_{max}-n}{n}\right)=z\log(K_D)-z\log([L])$$

As n_{max} has been previously obtained, a linear regression of the plot gives access to z and K_D:

$$\log\left(\frac{n_{max}-n}{n}\right)=f([L])$$

K_D = 14.81 nM

z = n_H (Hill coefficient) = 0.65

Computational methods

The following procedure does not provide accurate 3D structures of DGLs, which are suitable for docking applications. However, it does provide a good representation of their topology.

Previously reported characterization data[5], such as the mean branching ratio (BR) and the mean degree of polymerization (Dp) were used as a starting point to develop a homemade Python script. The branching ratio was defined as the ratio of the number of ε-branched Lys residues to the total number of residues (DP). From these parameters, it is possible to calculate the number of peptidic and isopeptidic bonds, as well as the number of free α- and free ε-amine functions in DGLs.

	Dp	BR	Free α	Free ε	Isopept. bonds	Pept. bonds
G1	8	0	1	8	0	7
G2	48	12.3	7	42	6	41
G3	123	24.0	31	93	30	92
G4	365	24.7	85	281	84	280

The purpose of the Python script was to “mimic” the synthesis of the DGLs through the manipulation of SMILE string chains. The starting point was the first-generation polymer G1 (a linear peptide of 8 residues):

[O-]C(=O)[C@@H](CCCCN)NC(=O)[C@@H](CCCCN)NC(=O)[C@@H(CCCCN)NC(=O)[C@@H](CCCCN)NC(=O)[C@@H](CCCCN)NC(=O)[C@@H](CCCCN)NC(=O)[C@@H](CCCCN)N

, to which some markers were added:

[O-]C(=O)[C@@H](CCCCN[E0])NC(=O)[C@@H](CCCCN[E1])NC(=O)[C@@H](CCCCN[E2])NC(=O)[C@@H](CCCCN[E3])NC(=O)[C@@H](CCCCN[E4])NC(=O)[C@@H](CCCCN[E5])NC(=O)[C@@H](CCCCN[E6])NC(=O)[C@@H](CCCCN[E7])N[X7]

, where [En] and [Xn] are ε and α positions that are available for further polymerization. Then, the sequential approach of the algorithm randomly replaced these markers with Lys building blocks, with respect to the BR and Dp parameters. The use of two different markers allows the discrimination of the protonation state of α- and ε-amines. At pH 7.8, we made the hypothesis that only the ε-amine residues of DGLs were protonated[6].

The SMILE string chains were then converted to 3D structures using the Discovery Studio software, and the equilibrium geometries were minimized using Spartan'10 at the MMFF level of theory in vacuum.[7] Finally, VMD[8] (version 1.9.1) and the APBS[9] plugin (version 1.3, default settings) allowed us to generate electrostatic potential maps.

The full source code of the script is available on request.

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