Supporting Information

Controlling Polymer Morphologies by Intramolecular and Intermolecular Dynamic Covalent Iron(III)/Catechol Complexation – From Polypeptide Single Chain Nanoparticles to Hydrogels

Marco Hebel\textsuperscript{1}, Jasmina Gačanin\textsuperscript{1}, Thorsten Lückerath, David Y. W. Ng*, Tanja Weil*

Marco Hebel, Jasmina Gačanin, Dr. Thorsten Lückerath, Prof. Dr. Tanja Weil
Institute of Inorganic Chemistry I, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany
E-mail: Tanja.Weil@uni-ulm.de

Jasmina Gačanin, Dr. Thorsten Lückerath, Dr. David Y. W. Ng, Prof. Dr. Tanja Weil
Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany
E-mail: david.ng@mpip-mainz.mpg.de, weil@mpip-mainz.mpg.de

\textsuperscript{1}Both authors contributed equally
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1. Index of Abbreviations

Mal 6-Maleimidohexanoic acid
AEEAc 8-amino-3,6-dioxaoctanoic acid
DOPA L-DOPA
HSA Human serum albumin
cHSA cationized HSA
dcHSA denatured cationized HSA
DCC N,N'-Dicyclohexylcarbodiimide

2. Experimental Procedures

2.1 Synthesis

Purchased Materials

PyBOP, Fmoc-Ala(Boc)-OH, Fmoc-Cys(Trt)-OH and Fmoc-Ala-Wang resin were purchased from Novabiochem®. N-ethyltriisopropylamine for synthesis (DIPEA) was obtained from Merck. Piperidine (≥ 99.5 % for peptide synthesis) and trifluoroacetic acid (TFA, ≥ 99.9 %) were obtained from Carl Roth. Dimethylformamide (DMF, peptide synthesis), diethyl ether and acetonitrile (HiPerSolv Chromanorm for HPLC-gradient grade) were purchased from VWR Chemicals Prolabo. Dimethylsulfoxid (DMSO, ACS reagent, ≥ 99.9 %) was purchased from Honeywell, Riedel-de Haën®. Syringe filters Chromafil®Xtra RC-20/13 (0.20 μm, 0.45 μm) were obtained
from Machery-Nagel. Vivaspin tubes (30 kDa MWCO) were purchased from Sartorius. Iron(III)-chloride (Roth: 98.5%). N-hydroxysuccinimide (98%) was purchased of Sigma-Aldrich. DCC (99%) was purchased of Sigma-Aldrich and 6-Maleimidohexanoic acid (98%) of TCI Chemicals. Fmoc-Dopa(acetonide)-OH (99%) and Fmoc-AEEAc-OH (99%) were purchased of Bachem.

2.1.1 Synthesis of C3-Maleimide

C3-Maleimide: Mal-AEEAc-DOPA-Ala-DOPA-Ala-DOPA-Ala

C3-maleimide peptide is synthesized using standard fmoc solid phase peptide synthesis (Fmoc-SPPS). Preloaded Ala-Wang resin was swelled overnight (DMF, 4°C) and used after 3 washes with fresh DMF (peptide grade).

The deprotection, coupling and final cleavage conditions of the Fmoc-protected amino acids are given in the protocol listed below.

Deprotection:

- Deprotection #1 (25% Piperidine in DMF, 3 min, R.T.)
- Deprotection #2 (25% Piperidine in DMF, 10 min, R.T.)
- Wash (4x DMF)
Coupling:

- Add reagents (5 equiv. amino acid, 5 equiv. PyBoP, 10 equiv. DIPEA)
- Microwave (8 min, 75°C)
- Wash (4x DMF)

The 6-maleimidohexanoic acid was coupled with the following conditions:

- Add 6-maleimidohexanoic acid N-hydroxysuccinimide ester (10 equiv.)
- Microwave (2 hours, 40 °C)
- Wash (4x DMF)

Final cleavage from the solid phase:

Treat the peptide, which is still bound to the solid phase with 95% TFA, 2.5 % TIPS, 2.5 % H2O, 2 h, R.T.

After the final cleavage, the peptide was precipitated by dropping the peptide solution into cold ether. Subsequently, it was purified with HPLC and characterized with mass spectrometry.

MALDI-ToF MS (H2O): m/z = 1107.45113 [M+H]^+, calc. mass: 1107.45.
2.1.2 Synthesis of 6-maleimidohexanoic acid N-hydroxysuccinimide ester

2.7 g (12.63 mmol) of 6-Maleimidohexanoic acid and 14.4 g (12.63 mmol) of $N$-hydroxysuccinimide were added to 25 ml of acetonitrile in an argon atmosphere. 2.6 (12.63 mmol) DCC was dissolved in 10 mL acetonitrile. The reaction mixture was cooled to 0 °C and the DCC solution was dropped slowly in. After stirring the mixture for 2 hours at 0 °C, the solution was stirred overnight at room temperature. The precipitated solid was filtered and washed with acetonitrile for 3 times. Then it was dried in vacuum to result in a pale brown/yellow material (yield: 93%).
2.1.3 Synthesis of cationized human serum albumin (cHSA)

cHSA was prepared according to the literature.\textsuperscript{[1]} HSA (600 mg, 9.1 µmol) was dissolved in 60 mL ethylendiamine (EDA) solution (2.5 M, pH = 4.75) at room temperature. Subsequently, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC, 3.07 g, 16 mmol) was added. The reaction was stirred for 2 h on an orbital shaker at room temperature. The reaction was stopped by addition of acetate buffer (4 mL, 4 M, pH = 4.75). Thereafter, the colorless solution was concentrated by ultrafiltration using Vivaspin 20 ultrafiltration tubes (30 kDa molecular weight cut-off (MWCO)) and washed two times with acetate buffer (100 mM, pH = 4.75) as well as five times with Milli-Q-water. After lyophilization (ALPHA 1-4LD Plus, Christ), the cHSA was obtained as a white solid (634 mg, 8.7 µmol, 96%). MALDI-ToF MS (H2O): m/z = 72651 [M+].
2.1.4 Synthesis of PEGylated cHSA (cHSA-PEG)

cHSA-PEG(2000)$_{22}$ was prepared according to the literature.$^{[1]}$ cHSA (130 mg, 1.8 μmol) was dissolved in 75 mL degassed phosphate-buffer (50 mM, pH 8.0). Thereafter, MeO-PEG(2000)-NHS ester (131.5 mg) was dissolved in 500 μL DMSO and introduced to the protein solution. The reaction mixture was stirred for 135 min at room temperature. The solution was concentrated and washed eight times with Milli-Q-water by ultrafiltration (Vivaspin 20 ultrafiltration tubes, 30 kDa MWCO). After lyophilization, the cHSA-PEG(2000)$_{22}$ was obtained as a white solid (190 mg, 1.76 μmol, 98%). In case the conjugated amount of PEG groups was found not to be sufficient, a second round of PEGylation was conducted with a suitable number of PEG equivalents.

MALDI-ToF MS (H2O): m/z = 115481 [M+].

![Mass spectrometry graph](image-url)
2.1.5 Synthesis of denatured cHSA-PEG (dcHSA-PEG)

Denatured **cHSA-PEG** was synthesized according to the literature.[2] First, aqueous urea-sodium phosphate buffer solution (50 mM PB, pH 7.4, 5 M urea) was degassed by holding vacuum for 0.5 min and adding argon to the solution. Then, **cHSA-(PEG2000)22** (20 mg, 0.18 umol) was solubilized in the solution and stirred for 15 min. Next, TCEP (tris(2-carboxyethyl)phosphine hydrochloride, 10.5 mg, 36.7 μmol, 200 eq.) was added to the mixture and stirred overnight under argon atmosphere. After that, the solution was ultrafiltrated (Vivaspin 20 ultrafiltration tubes, 30 kDa MWCO) with Milli-Q water for five times and lyophilized for 2 days to give a white solid (quantitative).

2.1.6 Synthesis of denatured cHSA-PEG-C3 (dcHSA-PEG-C3)

Denatured **cHSA-PEG** (21.92 mg, 0.20 umol) was solubilized in 0.5 mL DMSO. Then, C3-Maleimide (4.45 mg, 4.02 umol, 20 eq.) was solubilized in 50 uL DMSO and added to the solution. The reaction mixture was stirred for 26 hours with 1150 rpm. After that, n-propylmaleimide (5.60 mg, 40.2 umol, 200 eq., Sigma-Aldrich: 95%) was added to 50 uL DMSO and the resulting solution was injected to the mixture. The mixture was stirred for 16 h. After that, the solution was ultrafiltrated (Vivaspin 20 ultrafiltration tubes, 30 kDa MWCO) with Milli-Q water for five times and lyophilized for 2 days to give a white solid (quantitative). The SEC showed one a pure product and the addition of the catechol functionalities was proofed and determined by a chemosensor assay resulting in the functionalization of 18 catechol peptides (54 catechol groups) on **dcHSA-PEG-C3**.
Measurement of the amount of catechol functionalities

According to the literature, determination of the amount of catechol groups can be achieved by a specific binding of catechol functionalities to the chemosensor 2-anthracene boronic acid and a measurement of the fluorescence intensities.[3] First, seven solutions of 10 µL deHSA-PEG-C3 (15.38 µmol/L) were placed in wells of an UV Star® 384 microliter well-plate (Greiner bio-one). Then, a dilution series of C3-Maleimide solutions was mixed in DMSO and with 20 µL of volume, each. The concentrations of the solutions were listed in Table 1. 10 uL of each solution were also placed in other wells on the plate. After that 10 µL of 2-anthracene boronic acid in DMSO (2 mmol/L) was added to each well, following by the addition of 5 µL of phosphate buffer solution (pH 7.4, 300 mM).

| Solution | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| C3-Mal conc. [µmol] | 0   | 76.92 | 153.8 | 230.8 | 307.7 | 384.6 | 461.5 | 538.5 | 615.4 | 692.3 |

Table 1: Concentrations of the dilution series of C3-Maleimide for catechol chemosensor assay.
The solutions were measured in the fluorescence top reading mode with an excitation wavelength of 348 nm and an emission wavelength of 416 nm.

### 2.2 UV-Vis experiments

The backbone material dcHSA-PEG-C3 and the cross-linker Fe$^{3+}$ were dissolved as separate aqueous solutions (MilliQ), mixed in the required composition (Table 2) and the pH was adjusted to pH 4 by acidic acid/MilliQ and sodium hydroxide solution, to pH 7.4 by phosphate buffer/MilliQ (300 mM) and to pH 10 by sodium hydroxide solution. The absorption spectra were recorded on an Infinite® M1000 PRO microplate reader (Tecan). 25 µL of the different iron(III)-dcHSA-PEG-C3 solutions were placed in different wells of a transparent UV Star® 384 microliter well-plate (Greiner bio-one) and measured from 280 nm to 850 nm (Figure 1, Figure 2, Figure 3). dcHSA-PEG-C3 solutions were also measured within the same concentrations and with the different pH values and were subtracted of the signals, resulted with the mixture with iron(III).

| Volume [µL] of iron(III)-solution (100 mg/mL, 0.62 M) for 1 mL of dcHSA-PEG-C3 solution (1 mg/mL, 7 nM) | Equivalents of iron(III) in comparison to one equivalent of dcHSA-PEG-C3 |
|---|---|
| 0 | 0 |
| 0.13 | 10 |
| 0.38 | 30 |
| 0.56 | 45 |
| 1.12 | 90 |
| 2.24 | 180 |
| 4.48 | 360 |
**Figure 1:** UV-Vis absorption experiments of dcHSA-PEG-C3 with different amounts of iron(III) at pH 4.

**Figure 2:** UV-Vis absorption experiments of dcHSA-PEG-C3 with different amounts of iron(III) at pH 10.
Figure 3: UV-Vis absorption experiments of dcHSA-PEG-C3 with different amounts of iron(III) at pH 7.4.

2.3 DLS and SEC

2.3.1 Sample preparation for DLS and SEC

dcHSA-PEG-C3 solutions (0.1 mg/mL, 0.7 nM) were produced with MilliQ water/urea (8M) as a solvent. After that, the mixture was filtered by a syringe filter (pore size: 0.45 µm). An iron(III)-chloride solution (1 mg/mL, 6.15 mM) was mixed by adding water-free iron(III)-chloride to MilliQ water and subsequent vortexing. The filtered iron(III)-solution (filter pore size: 0.45 µm) was dropped in the dcHSA-PEG-C3 solutions (amounts in volume and equivalents of Fe$^{3+}$ in Table 3) and immediately mixed with the used eppendorf pipette tip and by vortexing (1200 rpm). The pH values were adjusted to pH 4 (acetic acid), to pH 7.4 (phosphate buffer, 50 mM) and to pH 10 (NaOH solution). The solutions were mixed for 30 min at 1150 rpm. The resulting solutions were filtered through a syringe filter (pore size: 0.45 µm).
Table 3: Volume of iron(III)-solution and equivalents of iron(III) for sample preparation for DLS and SEC.

| Volume [µL] of iron(III)-solution (1 mg/mL, 6.15 mM) for 1 mL of dcHSA-PEG-C3 solution (0.1 mg/mL, 0.7 nM) | Equivalents of iron(III) in comparison to one equivalent of dcHSA-PEG-C3 |
|---|---|
| 0 | 0 |
| 0.13 | 1 |
| 0.31 | 2.5 |
| 0.47 | 3.75 |
| 0.63 | 5.0 |
| 0.94 | 7.5 |
| 1.25 | 10 |
| 1.88 | 15 |
| 2.50 | 20 |
| 3.75 | 30 |
| 5.63 | 45 |
| 7.50 | 60 |
| 22.4 | 180 |

2.3.2 DLS and SEC measurement

The DLS measurements were conducted by a Malvern Pananalytical Zetasizer Nano ZS® device (Figure 4). The volumes of the samples were 1 mL, measured in disposable cuvettes.
**Figure 4:** Hydrodynamic size distributions obtained by DLS for dcHSA-PEG-C3 with different amounts of iron(III) at pH 7.4.

As size exclusion chromatography system an ÄKTA Purifier FPLC and Superdex 200 10/300 GL SEC column were used with an phosphate buffer (PB 100 mM, 8 M urea) as eluent and 0.3 mL/min as flow velocity.

**Table 4:** Nanoparticle size distributions and retention times, measured by DLS and SEC.

| Equiv. iron(III) | Size distribution [nm] by DLS | Retention time [min] by SEC |
|------------------|-------------------------------|-----------------------------|
|                  | 0  | 10  | 30  | 0   | 10  | 30  |
| pH 4             |    |     |     |     |     |     |
| 27 ± 0           | 27 ± 0.1 | 27 ± 0.1 | - | - | - |
| pH 7.4           | 25 ± 0.7 | 22 ± 0.7 | 19 ± 0.5 | 27.95 | 28.35 | 28.97 |
| pH 10            | 20 ± 0.1 | 23 ± 0.5 | 25 ± 0.4 | 28.42 | - | 29.92 |
2.4 AFM

2.4.1 Sample preparation for AFM

The initial sample (dcHSA-PEG-C3, 1 mM in 150 mM Phosphate buffer, pH 7.4 with 8 M urea) was diluted to 700 nM with phosphate buffer (50 mM, pH 7.4) and subsequently as a 30 μL sample applied onto the freshly cleaved mica substrate. The solution was left to incubate for 15 minutes in order to deposit the desired species on the mica substrate. After successful adsorption, the supernatant was removed and fresh phosphate buffer (250 μL) was added for the measurement.

2.4.2 AFM measurement

AFM measurements were conducted on a Dimension FastScan BioTM atomic force microscope from Bruker, which was operated in the PeakForce mode. AFM probes with a nominal spring constant of 0.25 Nm−1 were employed (FastScan-D, Bruker) for measurement in liquid. A circular mica disc (15 mm) was used as the substrate. Measurements were performed at scan rates between 0.8 and 2 Hz. Different areas of the mica substrate were scanned in order to ensure the integrity of the shown images. The images were finally processed by the software NanoScope Analysis 1.8.

2.5 Rheology

Rheological characterization was performed using a DHR3 rheometer (TA Instruments) which is provided with a temperature controller and a solvent reservoir to avoid dehydration of the hydrogel. The experiments were conducted using a parallel-plate geometry (8 mm plate, hydrogels of 30 μL volume, gap size of 0.45 – 0.60 mm). The experiments were performed at 25 °C.
2.5.1 Sample preparation for rheological measurements

The backbone material dcHSA-PEG-C3 and the cross-linker Fe$^{3+}$ were dissolved as separate aqueous solutions (MilliQ), mixed in the required composition (Table S2) and the pH was adjusted to pH 4 by acidic acid/MilliQ and sodium hydroxide solution, to pH 7.4 by phosphate buffer/MilliQ (300 mM) and to pH 10 by sodium hydroxide solution. The dcHSA-PEG-C3 solutions were mixed with concentrations of 4 wt% and 8w% (1.2 mg and 2.4 mg of backbone material in 30 µL gel), depending on the composition (Table 2). The FeCl$_3$ solutions were added with a concentration of 200 mg/mL. The exact volumes of the iron(III) solutions of the different compositions were also listed in Table 5.

| Table 5: List of produced hydrogels (H1-12) and their compositions with different amounts of iron(III), weight percentage of dcHSA-PEG-C3 and pH values. |
|---|
| H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | H11 | H12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Equiv. iron(III) | 45 | 90 | 180 | 180 | 45 | 90 | 180 | 180 | 45 | 90 | 180 |
| Vol. [iron(III) sol.] [uL] | 0.5 | 1 | 2 | 4 | 0.5 | 1 | 2 | 4 | 0.5 | 1 | 2 | 4 |
| wt% | 4 | 4 | 4 | 8 | 4 | 4 | 4 | 8 | 4 | 4 | 4 | 8 |
| pH 4 | | | | | | | | | | | |
| pH 7.4 | | | | | | | | | | | |
| pH 10 | | | | | | | | | | | |

2.5.2 Rheological measurements

To investigate the hydrogels mechanical properties, several different experiments were performed:

(i) Time sweep: The linear viscoelastic region was found to be in the range of 1% strain and 1 Hz frequency. Therefore, oscillatory time-sweep measurements were performed at a fixed strain of 1% and a fixed frequency of 1 Hz at 25 °C for 400 s.

(ii) Strain sweep: Oscillatory strain sweeps (0.01–1000%) were conducted at a fixed frequency of 1 Hz at 25 °C.
(iii) Frequency sweeps: Frequency sweeps (0.05–100 Hz) were performed at a fixed strain of 1% at 25 °C.

(iv) Thixotropy measurement: Combined measurements of an oscillatory strain sweep (0.01–1000%) at 25 °C with a fixed frequency of 1 Hz followed by an oscillatory time sweep measurement with a fixed strain of 0.1% and frequency of 1 Hz for 800 s at 25 °C were conducted to analyze the self-healing capacity of the hydrogels. The data was normalized to the mean storage modulus of the respective gel after the first strain sweep.

The following figures (all figures: G’ > G’’) point out the storage moduli (G’) and loss moduli (G’’) of the different gels, listed in Table 5.

**Figure 5, Figure 6, and Figure 7** show the time sweep experiments of the produced gels.

**Figure 5:** Time sweeps of different gels at pH 4.
Figure 6: Time sweeps of different gels at pH 7.4.
Figure 7: Time sweeps of different gels at pH 10.
Figure 8 contains the strain sweeps (G’ in black, G’’ in red).

Figure 8: Strain sweeps of different gels (G’ in black, G’’ in red).
Figure 9 contains the frequency sweeps ($G'$ in black, $G''$ in red).

Figure 9: Frequency sweeps of different gels (A: H8, B: H4, C: H6, D: H11, E: H12).
Figure 10 contains figures of the thixotropy measurements (G’ in black, G’’ in red).

**Figure 10**: Thixotropy measurements of different gels (G’ in black, G’’ in red).
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