Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Influence of the Chirality of Short Peptide Supramolecular Hydrogels in Protein Crystallogenesis.

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Materials & Methods

i) Description and synthesis of compounds 1 and 2

General Information:

Initiator™ (Biotage) was used to perform microwave irradiations.

Reagents were used as purchased from commercial sources unless otherwise specified.

LC-MS were carried out on an Agilent LCMSD 1100 equipped with an Supelco Discovery® C18 ODS, 5 μm, 50 mm x 4.6 mm i.d. column, flow rate: 1 mL min^{-1} as *per* Method 1.

LRES mass spectra were recorded using a VG Platform Quadrupole Electrospray Ionisation mass spectrometer, measuring monoisotopic masses.

HRES mass spectra were recorded using an LTQ-Orbitrap Classic (ThermoElectron, Germany) mass spectrometer.

^{1}H NMR and **^{13}C NMR** spectra were recorded were recorded on NMR 300, and 500 MHz spectrometers (Variant). Chemical shifts (δ), referenced to the residual protonated solvent as an internal standard, are quoted in ppm. Coupling constants (*J*) are reported in Hz. The compound **1** was isolated as pure sample and showed NMR spectra matching with the reported compound.[1]

RP-HPLC was performed on an Agilent 1100 Chemstation analytical system with a Supelco Discovery® C18 ODS, 5 μm, 50 mm x 4.6 mm i.d. column coupled to a Polymer Laboratories 100 ES evaporative light scattering detector. The following eluents were used: (A) H_2O + 0.1% TFA; (B) CH_3CN + 0.04% TFA; (C) H_2O + 0.1% FA; (D) MeOH + 0.1% FA. HPLC grade eluents were employed at a flow rate of 1 mL min^{-1} with samples prepared to a concentration of about 1 mg mL^{-1} and filtered prior to injection. The following method was used: *Method 1*: (10 min) 95% (C) to 5% (C) in (D) over 6 min, then 5% (C) in (D) over 3 min, then 5% (C) in (D) to 95% (C) over 0.05 min, then 95% (C) over 0.95 min, detection by UV at 230, 254, 260 and 280 nm and ELSD (evaporative light scattering detection).

Peptide synthesis:

Coupling reactions were carried out at 0.15M as final concentration

Method A - Solid phase peptide coupling conditions using carboxylic acids and free primary amines

Resin was previously swollen in DMF. Then, carboxylic acid (3.5 equiv) and HOBt (3.5 equiv) were dissolved in DMF. DIC (3.5 equiv) was added and the mixture stirred for 5

min before addition to the resin. The mixture was agitated for 1 h at 60 ºC to effect coupling. The resin was washed with DMF (x3), CH_2Cl_2 (x3).

Method B - N-terminal Fmoc removal

Fmoc removal was performed using 20% piperidine in DMF with two sequential treatments of 10 min. The resin was then filtered and washed with DMF (x3), CH_2Cl_2 (x3).

Method C - Cleavage of peptides from resin

Resin was swollen in DMF. TFA/TIS/DCM (90/5/5) was added and the resin agitated for 2 h. The TFA solution was removed, concentrated to *ca.* 0.5 mL and added to cold (4 °C) Et_2O in a centrifuge tube. The resulting precipitate was collected by centrifugation and washed with Et_2O (x3).

Method D - Intra-resin disulfide bond formation[2]

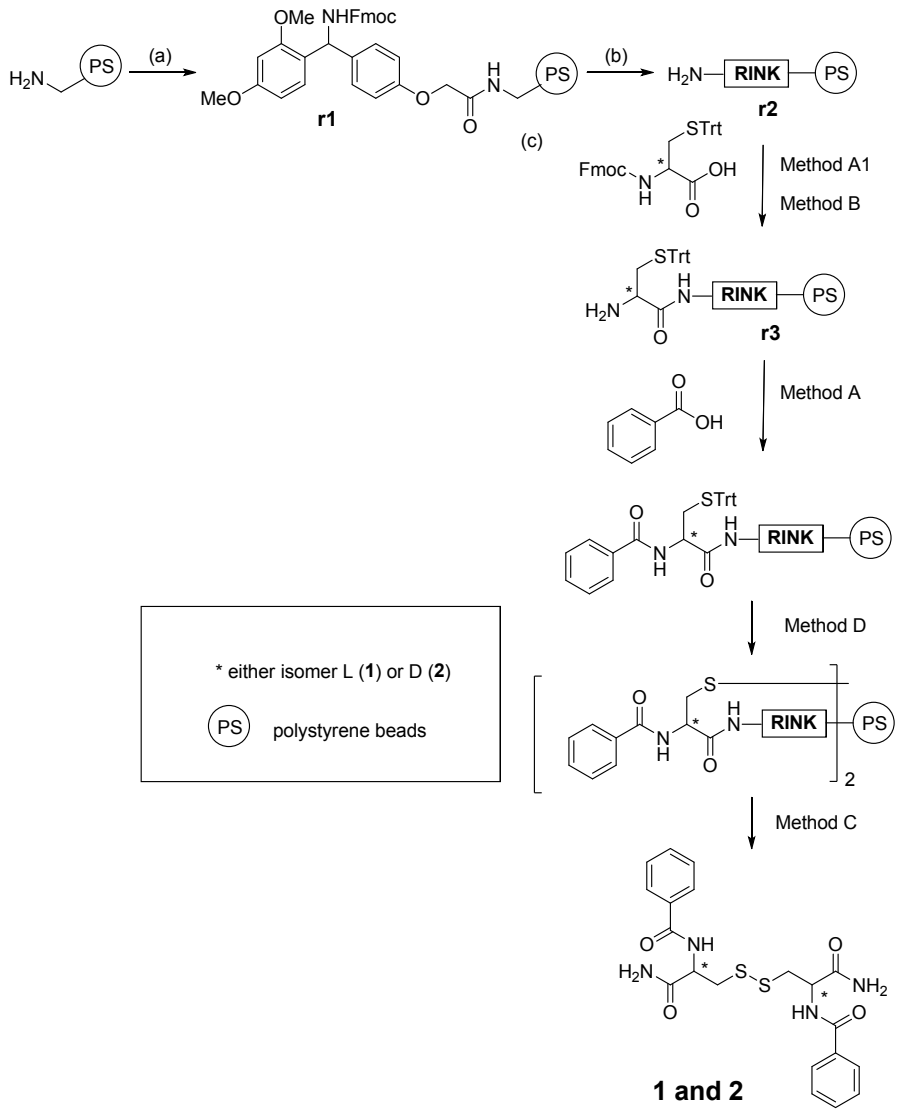
Trityl deprotection was carried out by 7 treatments (2 min each) of the ArCONH-Cys-S(Trt)-Rink amide-PS resin with a solution of DCM/TIS/TFA (93/5/2). Between each cleavage the resin was filtered and washed with DCM. The ArCONH-Cys(SH)-Rink amide resin was shaken with a solution of iodine (5 equiv) in DMF for 10 min. The resin was then washed with DMF (x 5), CH_2Cl_2 (x 5).

Quantitative Fmoc Test was carried-out as described in the literature[3]

To a known mass of dry Fmoc amino acid-resin (approximately 5 μmol with respect to Fmoc, (*e.g.* 5 mg) was added a solution of 20% piperidine in DMF (1 mL), and the mixture was shaken for 5 min.

Qualitative Ninhydrin Tests were carried-out as in literature[4]

Synthesis of compounds (1 and 2)



Fmoc-Rink-PS (r1)

Amino polystyrene resin (500 mg, 0.7 mmol, 1 equiv) was coupled to Fmoc-Rink amide linker (1500.8 mg, 2.8 mmol, 4 equiv) using *Method A*.

H-Rink-PS (r2)

Resin **r1** was Fmoc-deprotected using *Method B*.

H-Cys(Trt)-Rink-PS (r3)

Resin **r2** (1 equiv) was coupled to Fmoc-Cys(Trt)-OH (1435 mg, 2.45 mmol, 3.5 equiv) using *Method A1*. Qualitative ninhydrin test negative. Quantitative Fmoc test gave a loading of 0.608 mmol/g. Resin was deprotected using *Method C*. Qualitative ninhydrin test positive.

Resin **r3** (1 equiv) was coupled to carboxylic groups according to *Method A1 or A2 depending on the reagent*. Qualitative ninhydrin test negative. Resins were treated according to *Method D* and cleaved with *Method C*.

ii) Gelation Properties in Neat H_2O. Since **1** and **2** were not soluble in water at room temperature, they were microwave heated in a closed vial until complete dissolution. After a thoroughly screening we could conclude that the optimum conditions for gelation were heating at 180 °C for 5 minutes followed by an unassisted cooling to room temperature. Although compounds **1** to **2** can form gels in a ranges of concentrations, both of them form transparent and stable gels between 2 and 3 mM.[5]

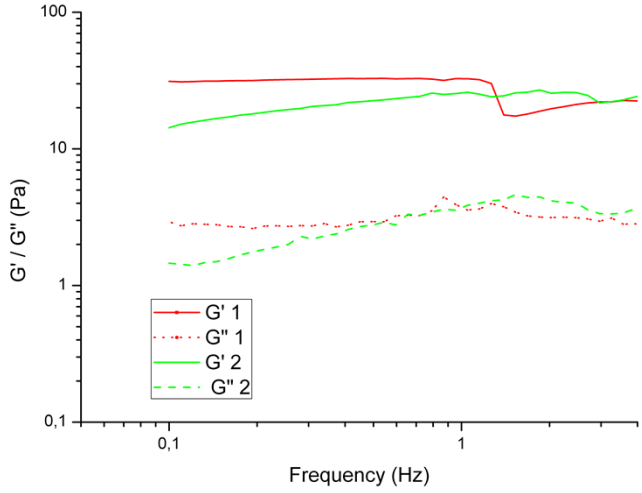
iii) TEM images. A drop of the hydrogel was placed on a 300-mesh copper grid. The sample was dried at room temperature for 30 min. Negative staining was obtained with a solution of 2% uranyl acetate in water for 2 min. Excess uranyl acetate was removed. Samples were viewed using a Carl Zeiss LIBRA 120 PLUS ESI (Electron Spectroscopic Image) electron microscope operating at 120 kV.

iv) Circular Dichroism. Spectra of **1** to **2** hydrogels were measured on a Jasco J-715 spectropolarimeter (Japan) equipped with a PTC-348WI temperature control unit, with 1 second accumulations every 0.5 nm and averaged over 5 acquisitions. Materials were prepared at a concentration of 1 mM and analyzed in a 0.1 cm quartz cell at 25 °C.

v) Rheology. Mechanical properties were carried out under shear stress using a controlled stress rheometer (Bohlin CS-10) using a cone–plate geometry (40 mm diameter, 4° cone angle) with a gap thickness of 0.150 mm. The stock hydrogels (2 mM) were shaken and 1.25 mL gently placed by means of a glass micropipette onto the lower plate of the rheometer. This plate have been preheated at 50 °C prior of placing the sample. Afterwards the sample was confined between both the cone and plate and maintained at 50 ºC during 5 min to favour the required homogeneity into the gel. Then, the temperature of the rheometer cell was slowly decreased down to 25 ºC in a time period of 30 min. After that, the measurements were carried out at 25.0 ± 0.1 °C while, to keep the sample hydrated, the samples were maintained in water vapor saturated atmosphere.

Dynamic or oscillatory tests were performed by applying a harmonic shear stress, recording the sinusoidal shear strain, where the shear stress applied depends on the frequency (*f,* Hz) and the shear amplitude, σ_0. From these experiments, we obtained the

so-called viscoelastic moduli, which completely characterize the viscoelasticity of the materials under shear: the elastic or storage modulus, G', and the viscous or loss modulus, G''. Thus, under oscillatory shear the G' and G'' allow a complete mechanical characterization of the material. With this purpose, amplitude sweeps oscillatory tests were carried out applying an oscillatory shear stress with a constant frequency while the stress amplitude, σ_0, was varied. The region in which the viscoelastic moduli (G', G'') do not depend on σ_0 is called the viscoelastic linear region (VLR). As a consequence, in the VLR, a pseudo-plateau is usually observed when G' and G'' are plotted as a function of σ_0. When the stress amplitude is higher than critical value, σ_c, the viscoelastic moduli depends, on σ_0: the material behaves as a non-linear viscoelastic system. In this work, the amplitude sweep experiments were conducted at a frequency of 1 Hz and the shear amplitude was varied between 0.06 and 50 Pa, being this interval wide enough for a fully sweeping of the VLR.



Frequency sweep measurements

vi) VT-NMR and T_2 measurements. The NMR spectra and relaxation measurements were recorded on an Agilent Direct Drive (500 MHz ^{1}H) and a variable-temperature accessory. Variable-temperature NMR measurements were recorded at 25, 50 and 70 ºC

successively with a minimum sample equilibration time of 30 min. The T_2 measurements were performed, with a relaxation delay of 40 s, using the CPMG pulse sequence with dephasing times short enough (0.4 ms) to suppress unwanted J-modulation of the spin-echoes. For T_2 relaxation measurements, a minimum of 12 points was obtained. The resulting curves could be fit satisfactorily to single exponential decays for the groups of measurable proton at 25 ºC.

vii) Differential Scanning Calorimetry. A drop of freshly prepared hydrogel **1** (17.08 mg) or **2** (24.42 mg) was placed on a DSC aluminium cell (40 μL) and sealed hermetically at room temperature. The gel was homogenized at 27 °C for 2 min and DSC run was recorded from 26 to 114 °C for **1** and 26 to 142 °C for **2** at 2 °C/min with air 50,0 mL/min. In a second experiment hydrogel **1** (28.13 mg) or **2** (22.31 mg) was homogenized at 100 °C for 1 min and DSC run was recorded from 97.8 to 25 °C at -2 °C/min with air 50,0 mL/min.

viii) Crystallization experiments. Lysozyme (chicken HEWL) and glucose isomerase (D-xylose-ketol-isomerase, GlucIsom) from S. rubiginosus were purchased as a lyophilized powder from Sigma (L6876) and as a crystal suspension from Hampton Research (HR7-100) respectively. The first one was dissolved in 50 mM Sodium Acetate pH 4.5. For the second one, crystals were dissolved in water and extensively dialyzed against 100 mM Hepes pH 7.0. *B. cereus* formamidase was expressed in *E. coli* BL21 (DE3) as a fusion protein with a C-terminal hexahistidine tag and purified in 50 mM Na_2HPO_4 and 300 mM NaCl buffer, from bacterial lysates by affinity chromatography (15 mL bed volume, GE Healthcare) and a Superdex 200 size exclusion column (GE Healthcare). In the second case, the buffer used was 25 mM Hepes pH 7.5, 200 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 5mM β-mercaptoetanol and 10% Glycerol and, the purification consisted in two affinity chromatography (15 mL bed volume, GE Healthcare) columns, one before cutting the His-Tag with TEV and another one after that. In both cases, based on SDS–PAGE experiments, the purity of the recombinant proteins was estimated to be greater than 95%. *Bacillus cereus* formamidase was dialyzed overnight against 20 mM Tris buffer pH 8.0 and

concentrated to 28 mg/mL using a Centricon centrifugation system with a 10 kDa molecular weight cutoff membrane.

Counter-diffusion technique with two layers configuration (2L)[6] was used to set-up crystallization experiments in Eppendorf tubes. Firstly the gel layer (50 µL) was prepared as mentioned above (**1** or **2** at a final concentration of 0.2 mM) or warming up the sol (agarose at a final concentration of 0.5% w/v) and let it set for one day. Them, 50 µL of the buffered protein solution was poured on top of the gel and allowed to diffuse at 20 ºC for 1 week. The solution was them removed and the remaining protein concentration measured spectrophotometrically. Finally 50 µL of precipitant was allowed to diffuse at 20 ºC. Final crystallization conditions are summarized in Table S1. The experiments were kept in incubators at 20 ºC and followed periodically with the help of an optical microscope.

Table S1. Initial protein and precipitant concentrations used with the 2L configuration of the gel counterdiffusion crystallization technique.

	Source	Protein concentration (mg/mL)	Precipitant composition
Lysozyme	Chicken HEWL	200	6% (*w/v*) NaCl, 0.05 M sodium acetate pH 4.5
Glucose isomerase	*S. rubiginosus*	110	1 M ammonium sulphate, 10 mM Tris pH 8.5
Formamidase	*B. cereus*	28	25% (*w/v*) PEG 4K, 0.2 M ammonium sulphate, 0.1 M sodium acetate pH 4.60

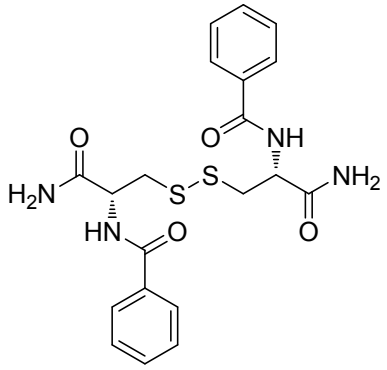
ix) X-ray data collection, analysis and structure determination. Crystal quality was evaluated by X-ray diffraction using synchrotron sources: beam-lines ID23-1 and BM30 of the European Synchrotron Radiation Facility (ESRF) and Xaloc beam-line of the ALBA Spanish Synchrotron. Shortly, after equilibration the precipitant solution was recovered and saved. Crystals were extracted from the gel using a pipetman (200 µL) with the tip-end cut and deposited over a plastic Petri dish. Drops of the recovered precipitant or the precipitant plus cryo-protectant (20% (v/v) glycerol) were deposited

nearby. Selected crystals were transferred to either the precipitant solution, for final cleaning, or the cryo-protectant solution with the help of a LithoLoop (Molecular Dimensions Inc.). Crystals were them flash cooled in liquid nitrogen and saved for data collection. For each series and protein data acquisition configuration: beam line, distance, exposure time and oscillation, was kept constant (see Tables S2 to S4 and Figures S1 to S4).

Data sets were indexed and integrated using XDS[7] and scaled with Scale implemented in Aimless of the CCP4 software package.[8] Coordinates from the glucose isomerase (PDB.ID 1AOD monomer A), from the same source organism *S. Rubiginosus*, were used as the search model for molecular replacement using Molrep.[9] Refinement was undertaken with Refmac[8] of the CCP4 program suite[10] followed by manual building and water inspection in Coot.[11] Previous deposition the final model was checked with Procheck.[12] Table 1 summarizes crystallographic data statistics and final model characteristics. The coordinates and the experimental structure factors have been deposited in the Protein Data Bank with entry code 4US6.

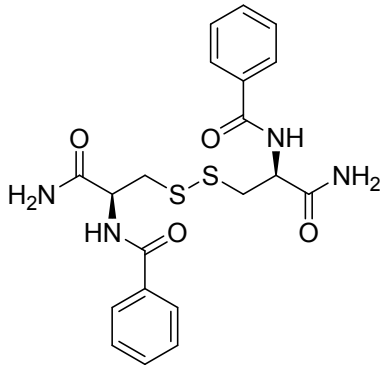
Supporting Results

N,N'-Di(benzoyl)-*L*-cysteine diamide (**1**)



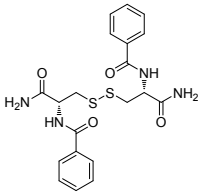
Yield: 95%; **RP-HPLC** (λ_{254}): 7.0 min (100%) (Method 1); ***m/z*** (ES^+): 447.0 $(M+H)^+$, 469.0 $(M+Na)^+$; **^{1}H-NMR** (DMSO-d_6, 300 MHz): δ 8.55 (d, 2H, *J* = 8.1 Hz, N*H*), 7.85 (d, 4H, *J* = 7.2 Hz, Ar*H*), 7.53-7.41 (m, 8H, NH_2 + 6Ar-*H*), 7.21 (s, 2H, NH_2), 4.73-4.65 (m, 2H, C*H*), 3.25 (dd, 2H, *J* = 4.2 Hz, *J* = 14.4 Hz, CH_2), 3.04 (dd, 2H, *J* = 10.2 Hz, *J* = 13.2 Hz, CH_2).

N,N'-Di(benzoyl)-*D*-cysteine diamide (**2**)

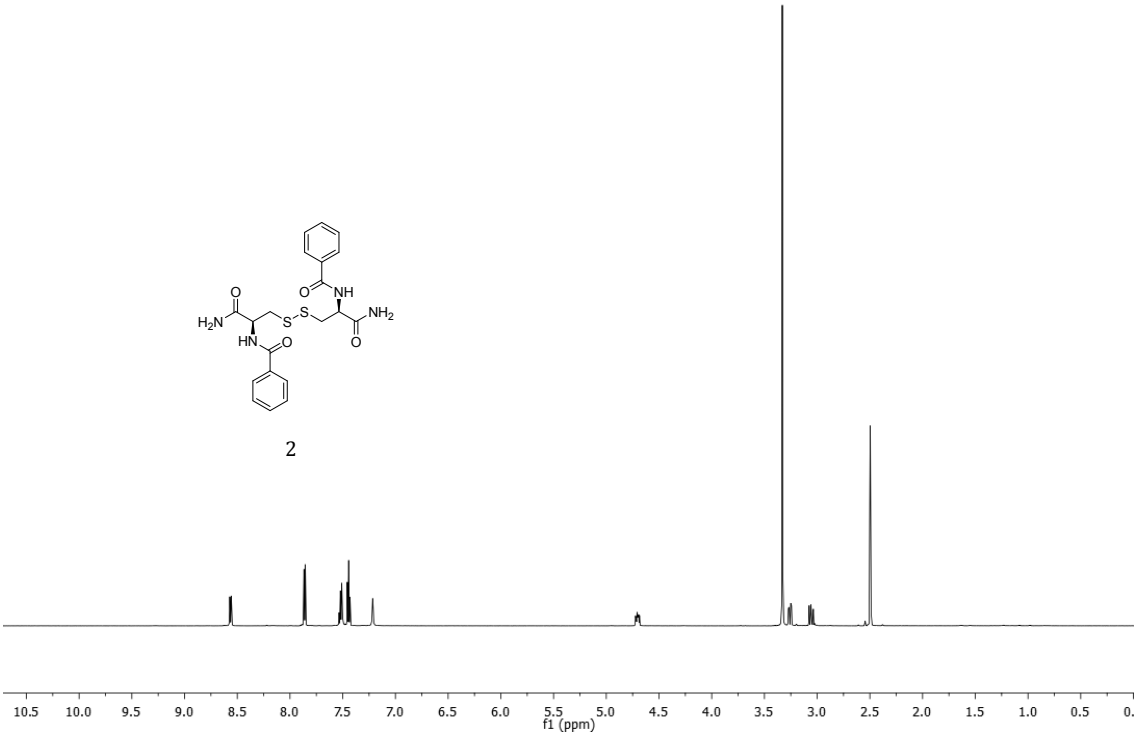


Yield: 95%; (Method 1); HRMS (ES$^+$) ***m/z*** calcd for $C_{20}H_{23}N_4O_4S_2$ $(M+H)^+$ 447.1161, found 447.1147; **^{1}H-NMR** (DMSO-d_6, 500 MHz): δ 8.56 (d, 2H, J = 7 Hz, N*H*), 7.86 (d, 4H, J = 7 Hz, Ar*H*), 7.53-7.51 (m, 4H, NH_2 + 2Ar-*H*), 7.45-7.43 (m, 4H, 4Ar-*H*), 7.21 (s, 2H, NH_2), 4.72-4.68 (m, 2H, C*H*), 3.25 (dd, 2H, J = 3.5 Hz, J = 11 Hz, CH_2), 3.05 (dd, 2H, J = 8.5 Hz, J = 11.5 Hz, CH_2). **^{13}C-NMR** (DMSO-d_6, 125 MHz): δ 172.016, 166.468, 133.989, 131.332, 128.156, 127.489, 52.474, 39.984 ppm.

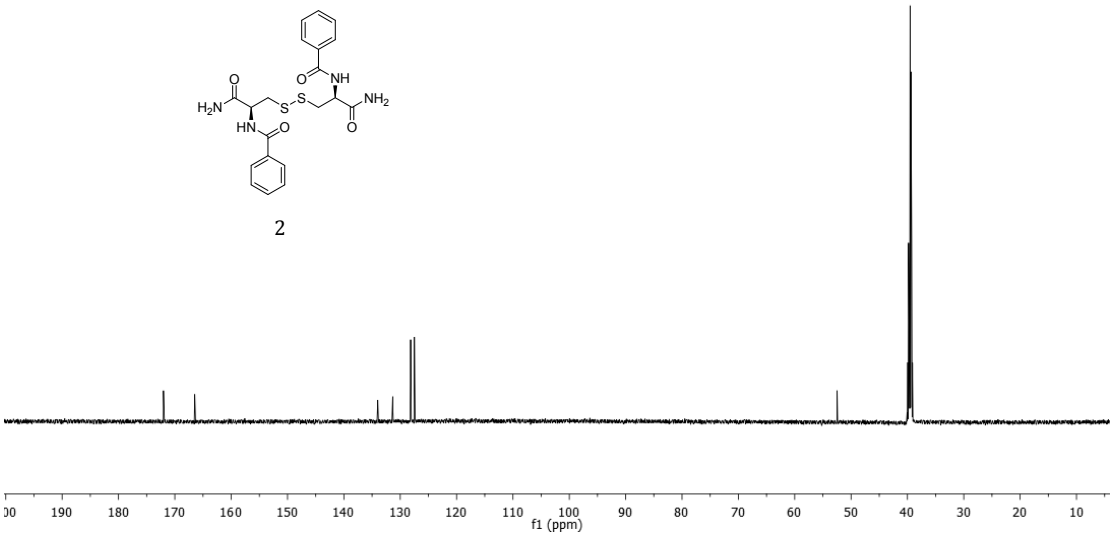
^{1}H and ^{13}C NMR spectra for 1 and 2



1

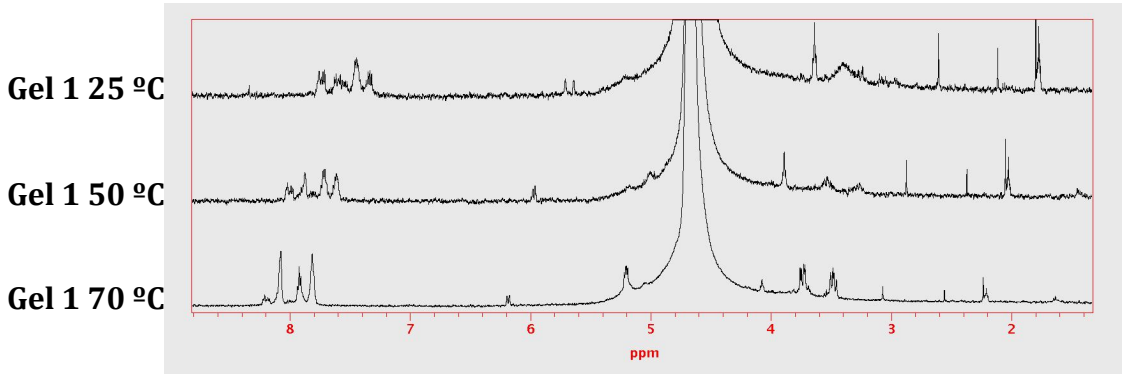

O
NH
H_2N
S
S
NH_2
HN
O
O
O
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.
f1 (ppm)

2

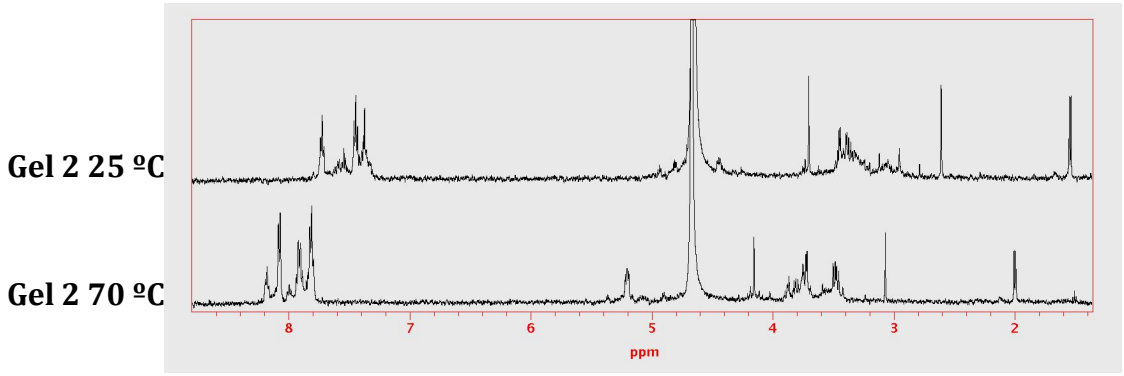

O
NH
H_2N
S
S
NH_2
HN
O
O
O
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

2

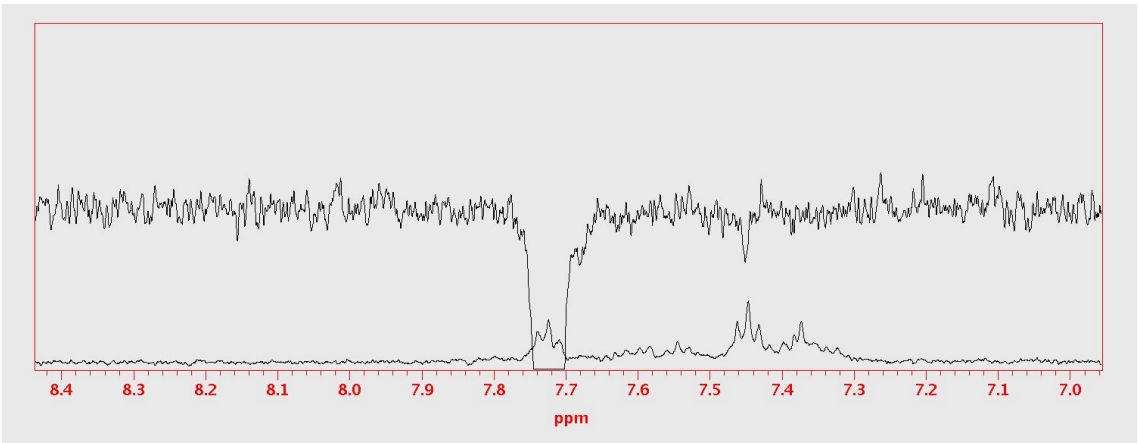
VT-NMR for hydrogel 1 in D_2O



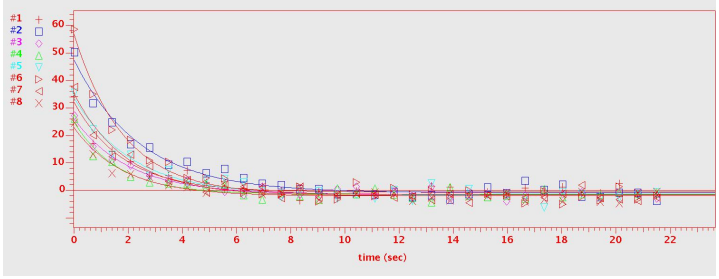
VT-NMR for hydrogel 2 in D_2O



nOe experience of hydrogel 2

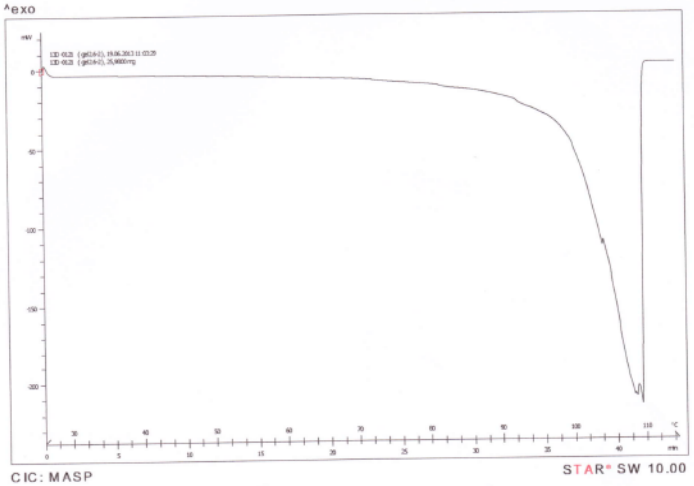


Transverse Magnetic Relaxation (T_2) of hydrogel 2 fitting to single exponential decays



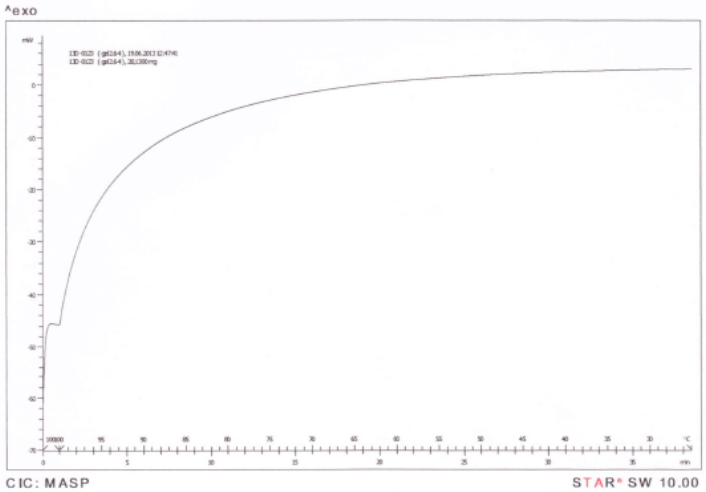
DSC scans for hydrogel 1

Evaluation:



Curve:	**13D-0121 (gel2.6-2), 19.06.2013 11:03:29**
Sample:	13D-0121 (gel2.6-2), 25,9800 mg
Sample Holder:	Aluminum Standard 40ul Weight : 0 Material: Aluminium
Method:	DSC Normal 500C 2C/min 50A 2m dt 1,00 s [1] (27,0 °C, 2,00 min Air 50,0 ml/min) [2] 26,0..500,0 °C, 2,00 K/min Air 50,0 ml/min Synchronization disabled
Module:	DSC 1/700/1218- Cal, 01.06.2011 11:57:57
User:	MASP

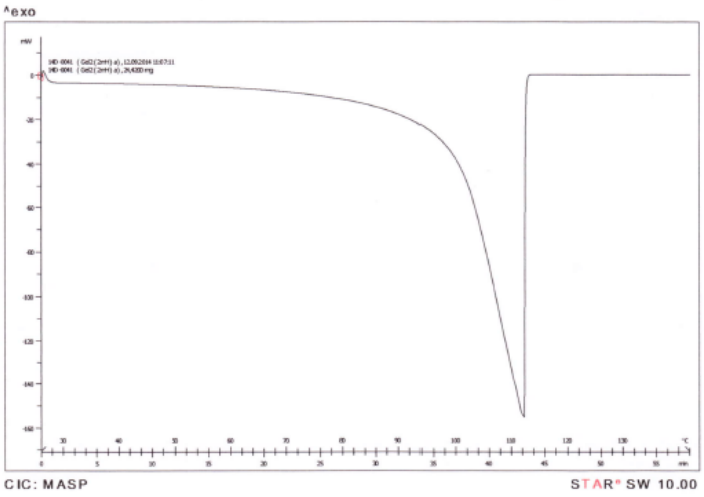
Evaluation:



Curve:	**13D-0123 (gel2.6-4), 19.06.2013 12:47:41**
Sample:	13D-0123 (gel2.6-4), 28,1300 mg
Sample Holder:	Aluminum Standard 40ul Weight : 0 Material: Aluminium
Method:	Cienfuegos100-25 dt 1,00 s [1] 100,0 °C, 1,00 min Air 50,0 ml/min [2] 100,0..25,0 °C, -2,00 K/min Air 50,0 ml/min Synchronization enabled
Module:	DSC 1/700/1218- Cal, 01.06.2011 11:57:57
User:	MASP

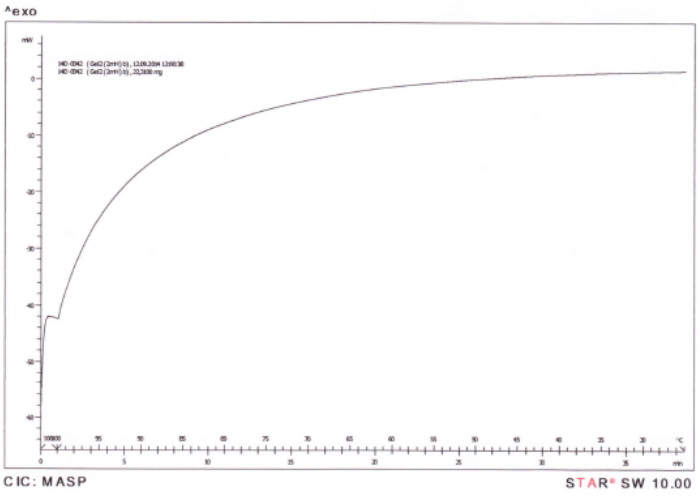
DSC scans for hydrogel 2

Evaluation:



Curve:	**14D-0041 (Gel2 (2mH) a), 12.09.2014 11:07:11**
Sample:	14D-0041 (Gel2 (2mH) a), 24,4200 mg
Sample Holder:	Aluminum Standard 40ul Weight : 0 Material: Aluminium
Method:	DSC Normal 500C 2C/min 50A 2m dt 1,00 s [1] (27,0 °C, 2,00 min Air 50,0 ml/min) [2] 26,0..500,0 °C, 2,00 K/min Air 50,0 ml/min Synchronization disabled
Module:	DSC 1/700/1218- Cal, 01.06.2011 11:57:57
User:	MASP

Evaluation:



Curve:	**14D-0042 (Gel2 (2mH) b), 12.09.2014 12:00:38**
Sample:	14D-0042 (Gel2 (2mH) b), 22,3100 mg
Sample Holder:	Aluminum Standard 40ul Weight : 0 Material: Aluminium
Method:	Cienfuegos100-25 dt 1,00 s [1] 100,0 °C, 1,00 min Air 50,0 ml/min [2] 100,0..25,0 °C, -2,00 K/min Air 50,0 ml/min Synchronization enabled
Module:	DSC 1/700/1218- Cal, 01.06.2011 11:57:57
User:	MASP

Table S2. Summarized the data collection conditions and final statistical values of lysozyme crystals (Set-1) obtained in gel **1, 2** and in agarose (data in brackets correspond to high resolution shell) at Xaloc beam-line (ALBA) at 100 K.

Gel type	Agarose	Agarose	Agarose	**1**	**1**	**1**	**2**	**2**	**2**
Data Acquisition									
ESRF Beam-line	ALBA	ALBA	ALBA	ALBA	ALBA	ALBA	ALBA	ALBA	ALBA
Detector type	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M
Wavelength (Å)	0.980	0.980	0.980	0.980	0.980	0.980	0.980	0.980	0.980
Distance (mm)	160.35	160.35	160.35	160.35	160.35	160.35	160.35	160.35	160.35
Exposure time (ms)	0.2seg	0.2seg	0.2seg	0.2seg	0.2seg	0.2seg	0.2seg	0.2seg	0.2seg
Oscilation (º)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Data Statistics									
Space group	P 4_3 2_1 2	P 4_3 2_1 2	P 4_3 2_1 2	P 4_3 2_1 2	P 4_3 2_1 2	P 4_3 2_1 2	P 4_3 2_1 2	P 4_3 2_1 2	P 4_3 2_1 2
Unit cell									
a=b, c (Å)	*77.27, 37.92*	*77.25, 37.9*	*77.36, 37.78*	*77.32, 38*	*77.31, 37.85*	*77.57, 37.69*	*77.35, 38.02*	*77.31, 37.89*	*77.29, 37.96*
Resolution (Å)	38.64-1.15	38.62-1.15	38.68-1.15	38.66-1.15	37.85-1.15	37.69-1.15	38.67-1.15	38.66-1.15	38.65-1.15
(High shell)	(1.17-1.15)	(1.17-1.15)	(1.17-1.15)	(1.17-1.15)	(1.17-1.15)	(1.17-1.15)	(1.17-1.15)	(1.17-1.15)	(1.17-1.15)
Unique reflections	41364 (1992)	41317 (1997)	41322 (2062)	41506 (2001)	996262 (1989)	41441 (2063)	41562 (2015)	41378 (1998)	41429 (2006)
R-merge * (%)	3.2 (40.7)	8.0 (67.4)	3.9 (39.9)	3.9 (24.7)	5.3 (24.6)	6.6 (77.8)	3.7 (15.6)	3.6 (20.3)	3.5 (15.6)
I/σ(I)	51.9 (9.0)	22.0 (4.5)	43.6 (9.1)	49.2 (12.6)	37.5 (11.8)	27.6 (4.8)	56.4 (18.8)	53.3 (15.4)	57.2 (18.1)
Completeness (%)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)
Redundancy	24.4 (23.0)	23.7 (23.2)	24.6 (23.8)	24.7 (23.8)	24.1 (23.7)	24.5 (23.7)	24.7 (23.7)	24.7 (23.9)	24.3 (23.0)
B-factor ($Å^2$)	10.6	9.7	10.3	10.2	9.6	10.7	10.2	10.2	10.1
Mosaicity	0.22	0.22	0.23	0.14	0.17	0.20	0.11	0.12	0.16

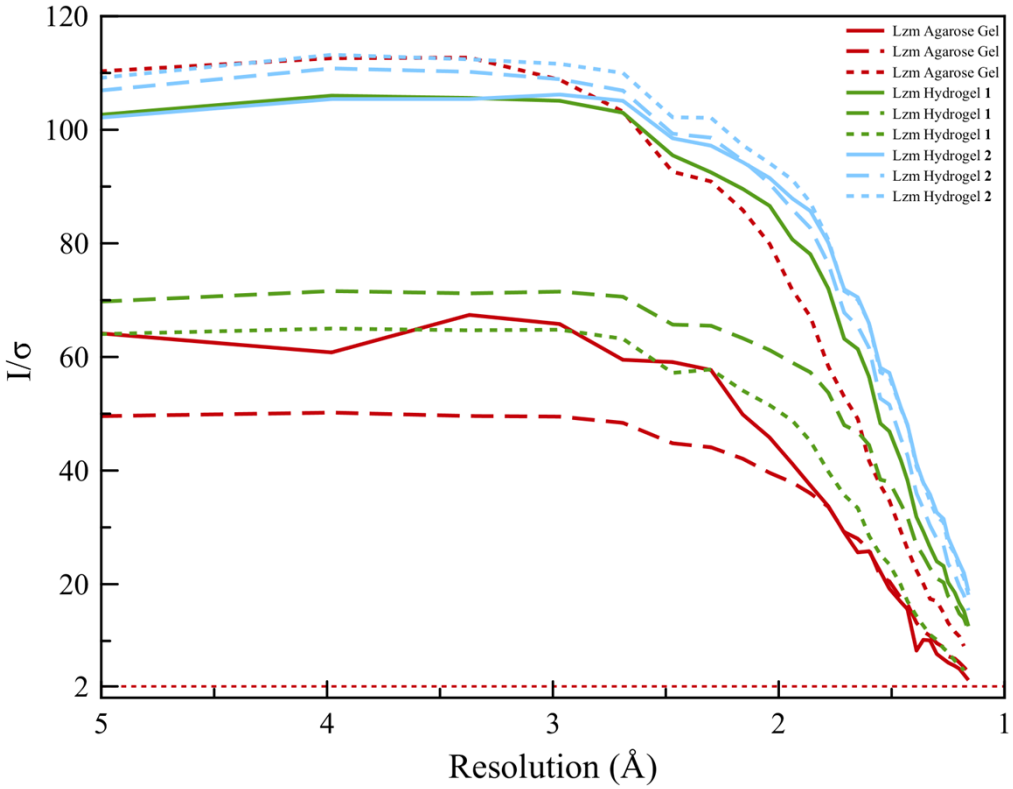


Figure S1. Shown signal to noise ratio as a function of the resolution of X-ray data collected from lysozyme crystals grown in hydrogels **1**, **2** and in agarose at beam-line Xaloc (ALBA) (complete statistical analysis in Table S2).

Table S3. Summarized the data collection conditions and final statistical values of lysozyme crystals (Set-2 and Set-3) obtained in gel **1** and in agarose (data in brackets correspond to high resolution shell).

	SET 2				SET 3			
Gel type	Agarose	Agarose	**1**	**1**	Agarose	Agarose	**1**	**1**
Conditions	Naked	20% Glycerol	Naked	20% Glycerol	Naked	20% Glycerol	Naked	20% Glycerol
Data Acquisition								
ESRF Beam-line	ID23-1	ID23-1	ID23-1	ID23-1	BM30	BM30	BM30	BM30
Detector type	Pilatus	Pilatus	Pilatus	Pilatus	ADSC Q315	ADSC Q315	ADSC Q315	ADSC Q315
Wavelength (Å)	0.972	0.972	0.972	0.972	0.979	0.979	0.979	0.979
Distance (mm)	158.99	158.99	158.99	158.99	151.43	151.43	151.43	151.43
Exposure time (ms)	0.037min	0.037min	0.037min	0.037min				
Oscilation (°)	0.1	0.1	0.1	0.1	1.0	1.0	1.0	1.0
Data Statistics								
Space group	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$	$P4_32_12$
Unit cell								
a=b, c (Å)	79.00, 37.29	78.69, 36.97	78.74, 37.33	78.59, 36.99	79, 37.46	79.01, 37.11	79.03, 37.48	78.66, 37.2
Resolution (Å)	39.50-1.20	39.35-1.20	39.37-1.20	39.29-1.20	39.50-1.6	10.30-1.60	39.52-1.60	39.33-1.60
(High shell)	(1.22-1.20)	(1.22-1.20)	(1.22-1.20)	(1.22-1.20)	(1.63-1.60)	(1.63-1.60)	(1.63-1.60)	(1.63-1.60)
Unique reflections	37449 (1794)	36915 (1788)	37314 (1810)	36835 (1784)	15910 (775)	15999 (786)	15994 (774)	15367 (755)
R-merge * (%)	3.6 (39.8)	6.0 (51.1)	3.9 (30.8)	3.7 (23.4)	4.5 (27.1)	59.9 (62.6)	4.1 (17.8)	6.1 (26.8)
I/σ(I)	31.1 (6.2)	17.3 (3.7)	33.3 (7.8)	34.0 (9.3)	25.2 (5.6)	3.0 (1.7)	28.7 (8.1)	20.3 (5.8)
Completeness (%)	99.9 (99.5)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	98.6 (99.9)	99.6 (100.0)	98.9 (100.0)	96.9 (98.8)
Redundancy	12.2 (12.5)	12.5 (12.3)	12.6 (12.5)	12.3 (12.4)	6.9 (7.8)	11.3 (11.1)	6.8 (7.8)	7.3 (7.7)
B-factor ($Å^2$)	11.3	10.6	11.3	11.5	10.4	7.6	9.4	8.5
Mosaicity	0.10	0.18	0.06	0.11	0.28	0.53	0.32	0.32

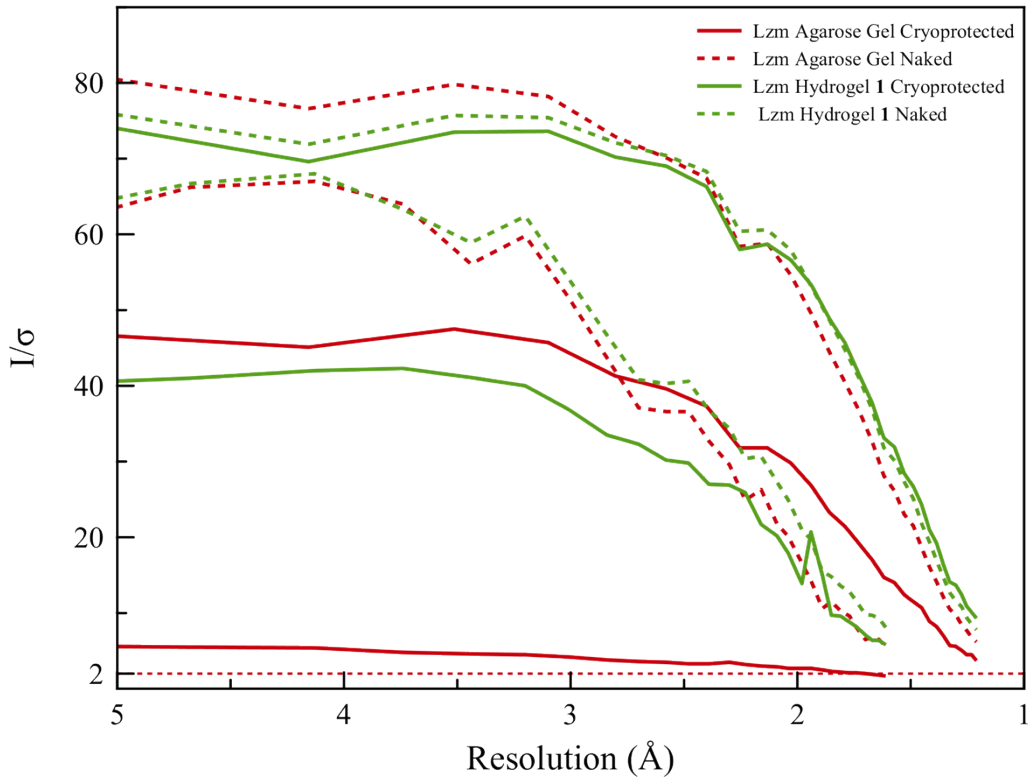


Figure S2. Shown signal to noise ratio as a function of the resolution of X-ray data collected from lysozyme crystals grown in gel at beam-line ID23-1 and BM30 (complete statistical analysis in Table S3).

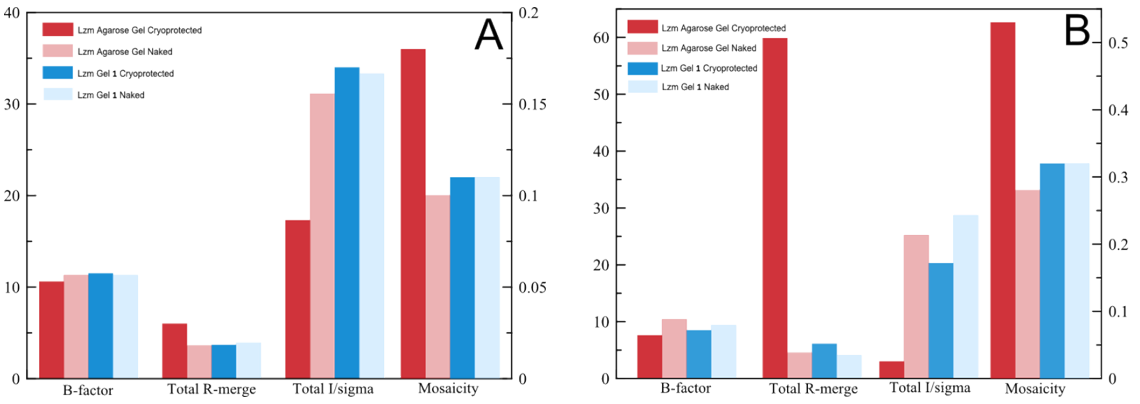


Figure S3. Quality indicators of lysozyme crystals grown in hydrogel **1** and agarose from cryo-protected and naked crystals. Values were obtained from single crystals measured at ID29 (A) and BM30 (B). Data sets were collected at 100 K keeping constant all data acquisition configuration set-up and analysis parameters. Complete statistics are given in the supplementary information.

Table S4. Summarized the data collection conditions and final statistical values of glucose isomerase crystals grown in gel **1**, **2** and in agarose (data in brackets correspond to high resolution shell).

Gel type	Agarose	Agarose	**1**	**1**	**2**	**2**
Data Acquisition						
ALBA Beam-line	Xaloc	Xaloc	Xaloc	Xaloc	Xaloc	Xaloc
Detector type	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M	PILATUS 6M
Wavelength (Å)	0.980	0.980	0.980	0.980	0.980	0.980
Distance (mm)	216.99	216.99	216.99	216.99	216.99	216.99
Exposure time (ms)						
Oscilation (º)	0.25	0.25	0.25	0.25	0.25	0.25
Data Statistics						
Space group	*I 2 2 2*	*I 2 2 2*	*I 2 2 2*	*I 2 2 2*	*I 2 2 2*	*P* 2_1 2_1 2*
Unit cell						
a, b, c (Å)	93.03,98.77,102.1	93.05, 97.82, 102.4	92.85, 97.24, 102.2	92.98, 96.99, 102.2	93.46, 98.07, 102.5	86.00, 93.68, 99.22
Resolution (Å)	70.77-1.30	46.53-1.30	48.62-1.30	46.49-1.30	49.03-1.6	49.61-1.32
(High shell)	(1.32-1.30)	(1.32-1.30)	(1.32-1.30)	(1.32-1.30)	(1.63- 1.60)	(1.32 – 1.30)
Unique reflections	114479 (5620)	114422 (5634)	113124 (5517)	113121 (5546)	62264 (3053)	196510 (9670)
R-merge * (%)	10.6 (59.5)	18.7 (73.9)	17.3 (82.6)	9.9 (58.0)	20.8 (69.8)	8.1 (37.4)
I/σ(I)	12.5 (3.0)	8.6 (2.9)	8.6 (2.4)	14.9 (4.1)	8.4 (2.9)	19.9 (7.0)
Completeness (%)	100.0 (100.0)	100.0 (100.0)	99.9 (99.8)	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)
Redundancy	11.6 (9.4)	11.8 (9.9)	12.0 (10.3)	12.8 (12.5)	12.3 (11.4)	12.9 (12.7)
B-factor ($Å^2$)	10.6	7.4	8.0	8.2	9.3	6.8
Mosaicity	0.38	0.40	0.35	0.29	0.41	0.16

* Second polymorph of glucose isomerase crystal grown in gel type **2**.

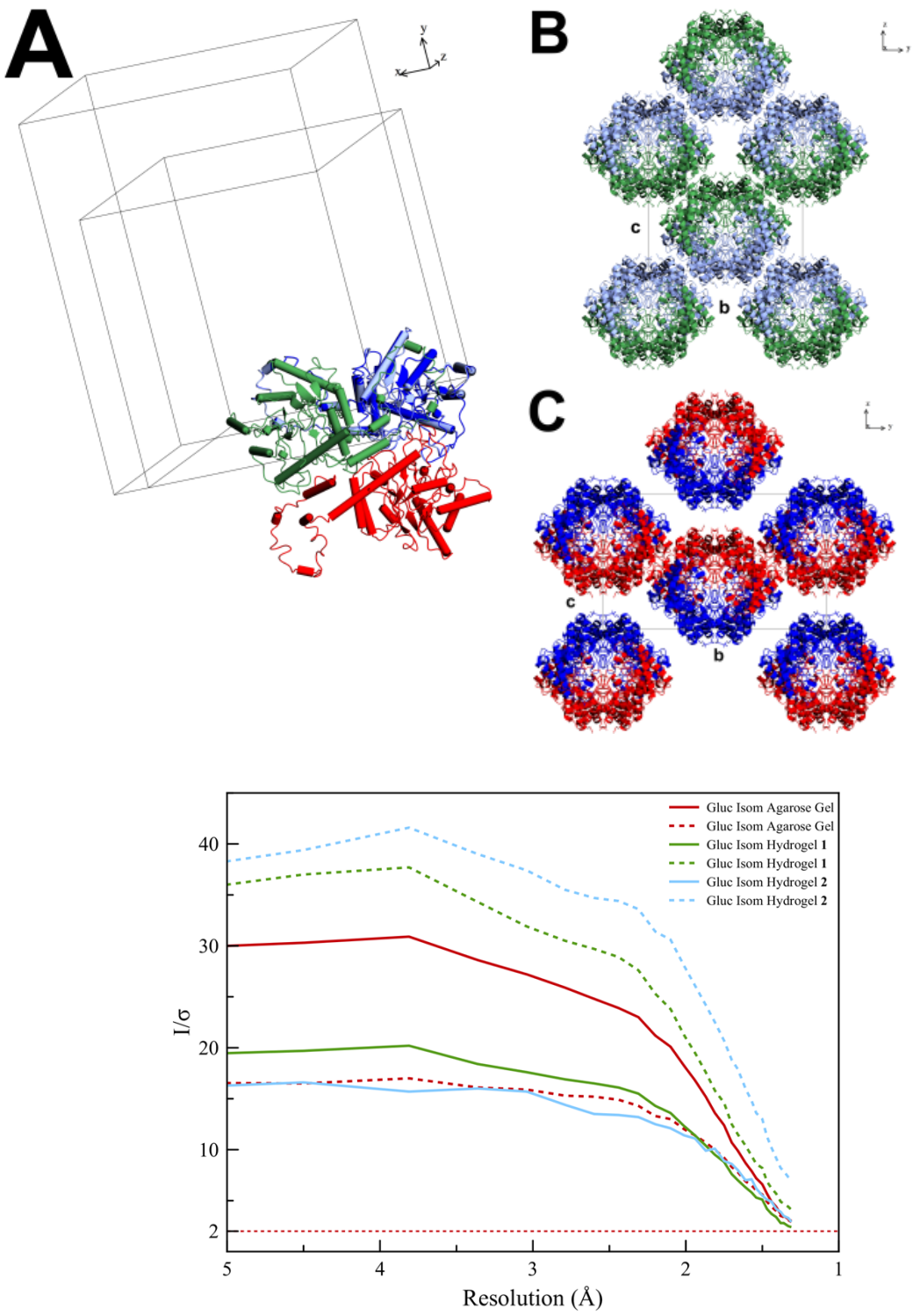


Figure S4. Glucose isomerase crystal packing in the already reported (PDB ID. 1OAD) and the new P21212 polymorphs (PDB ID. 4US6). A) shows the superposition of chain A (blue for 1OAD and light blue for 4US6) and chains B (red for 1OAD and green for 4US6) place at the origin of the unit cells. B) (this work) and C) (PDB ID. 1OAD) show the packing of both polymorphs along the a axis. Lower part shown the signal to noise ratio as a function of the resolution of X-ray data collected from glucose isomerase crystals grown in hydrogels **1**, **2** and in agarose at beam-line Xaloc (ALBA) (complete statistical analysis in Table S4).

Table S5. Data collection and refinement statistics of the $P\,2_12_12$ of glucose isomerase from a crystal grown in hydrogel **2**.

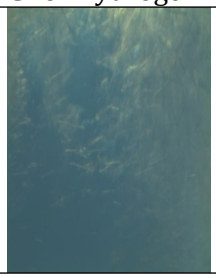
PDB identifier	4US6
Data collection	
Beam line	Xaloc (ALBA)
Space Group	P 21 21 2
Cell dimensions	
a, b, c (Å)	85.99, 93.67, 99.21
ASU	2
Resolution (Å)	99.21 - 1.2 (1.243 - 1.2)
R_{sym} (%)	9.05 (56.57)
I/σ_I	16.86 (3.00)
Completeness (%)	99.37 (93.88)
Unique reflections	247660 (23186)
Multiplicity	12.0 (7.0)
CC(1/2)	0.999 (0.864)
Refinement	
Resolution (Å)	99.21 - 1.2
R_{work}/R_{free} (%)	10.65 / 13.24
No. atoms	7687
Protein	6688
Ligands	61
Water	937
Average B-factors (Å^2)	13.5
R.m.s deviations	
Bond lengths (Å)	0.019
Bond angles (^{0})	1.93
Ramachandran (%)	
Favored	97.0
Outliers	0.24

Values in parentheses are for highest-resolution shell.

Table S6. Data collection statists of X-ray data collected form crystal of formamidase grown in peptide hydrogel **2** and in solution (Data in brackets correspond to high resolution shell).

	Gel	Solution
Beamline	Xaloc (ALBA)	Xaloc (ALBA)
Space group	C 1 2 1	C 1 2 1
Unit cell parameters (Å)	a=141.5,b=149.8, c=97.82	a=143.8, b=150.2, C=98.26
Wavelength (Å)	0.979493	1.033198
Resolution range (Å)	48.35–1.40 (1.42-1.40)	48.57-1.78 (1.81-1.78)
Total number of reflections	2654635 (131024)	561595 (10430)
Total unique reflections	381612 (18431)	182818 (5757)
R-merge (%)	7.7 (99.1)	7.6 (60.1)
I/σ(I)	13.7 (1.9)	10.7 (1.7)
Completeness (%)	97.0 (94.9)	93.1 (59.2)
Redundancy	7.0 (7.1)	3.1 (1.8)
B-factor (Å^2)	12.0	16.0
Mosaicity	0.1	0.1

Table S7. Summarized the crystallization results of formamidase from *B. cereus* obtained by counterdiffusion in gel (agarose, hydrogel **1** and **2**) in a two-layer configuration. Condition C18 (GCB-GSK, Triana S&T, Granada, Spain) in hydrogel **2** produced the crystal diffracting X-ray to 1.4 Å.

C3$^+$ Agarose	**C3**$^+$ Hydrogel **1**	**C18**† Hydrogel **2**	**C20**$^\#$ Agarose	**C20**$^\#$ Hydrogel **1**
				

$^+$**C3**: 20% PEG 8000, magnesium acetate 0.2M, sodium cacodylate 100 mM pH 6.50.
†**C18**: 25% PEG 4000, ammonium sulfate 0.2 M, sodium acetate 100 mM pH 4.60.
$^\#$**C20**: 30% PEG 8000, sodium acetate 0.2 M, sodium cacodylate 100 mM pH 6.50.

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