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Supplementary information

Tuning Alginate β-Lactoglobulin Complex Coacervation by Modulating pH and Temperature

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1. Methods

1.1 Confocal Microscopy

Alginates were labelled with fluorescein and β-Lg was labelled with *Abberior* STAR RED. As both types of labelling target the interacting groups (carboxylate on alginate and lysine on β-Lg) labeling degree was kept at 1 (number of labels / molecule) for β-Lg and 5 for alginate. 0.67 µM labelled alginate (0.2–0.3 mg/mL) was mixed with 27 µM (0.5 mg/mL) labelled β-Lg in 10 mM universal buffer (Brooke et al., 2015) pH 4.0, 75 mM NaCl and incubated at 25°C, 55 or 95°C for 5 min. Samples were cooled and transferred to the microscope plate for imaging. Images were obtained using a total internal reflection fluorescence microscope (IX 83, Olympus, Tokyo, Japan) using a EMCCD camera (ImagEM X2, Hamamatsu, Hamamatsu City, Japan) and an oil immersion 100× objective (UAPON 100XOTIRF, Olympus, Tokyo, Japan) equipped with the detectors and filter sets for monitoring of fluorescein fluorescence (excitation, 488 nm; emission, 517 nm) and *Abberior* STAR RED (excitation, 630 nm; emission, 655 nm).

2. Figures

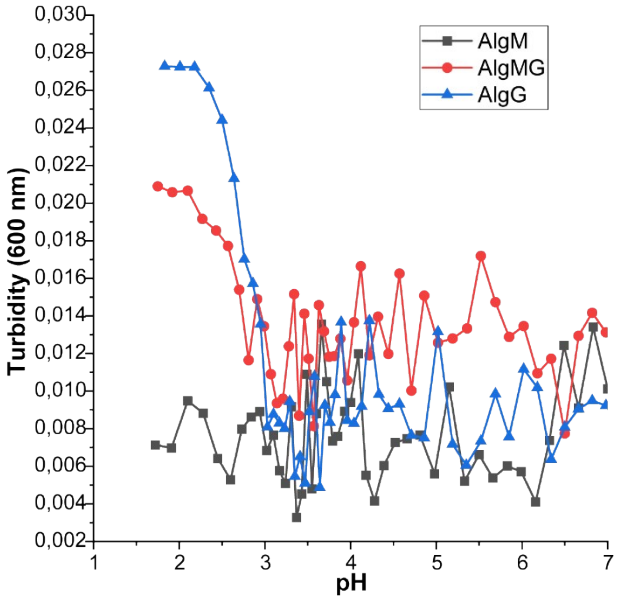


Fig. S1. Phase diagrams of 0.67 µM alginates (0.2–0.3 mg/mL) followed by turbidity (left panel) as a function of pH. AlgM β-Lg (black), AlgMG (red), AlgG (blue).

Table S1. Interaction parameters for alginate-β-Lg complexation in bicine (ΔH_{ion} = 11.4 kJ/mol), citrate (ΔH_{ion} = 4.0 kJ/mol), phosphate (ΔH_{ion} = –8.0 kJ/mol) at pH 2.65 and 20 mM oxalate (ΔH_{ion} = –7.0 kJ/mol), acetate (ΔH_{ion} = –0.4 kJ/mol) , citrate (ΔH_{ion} = 2.2 kJ/mol) at pH 4.0 derived from ITC (Figs. S4 and S5) (sample size = 3).

	K_d (µM)	n	ΔH (kJ/mol)	-T*ΔS (kJ/mol)	ΔG (kJ/mol)
Bicine	0.1 ± 0.3	10.8 ± 0.3	-145.7 ± 3.4	106.5 ± 3.0	-39.2 ± 0.6
Citrate	0.3 ± 0.3	14.0 ± 0.4	-110.9 ± 2.8	73.3 ± 4.7	-37.6 ± 2.0
Phosphate	0.4 ± 0.3	15.3 ± 1.7	-73.8 ± 3.2	36.9 ± 2.2	-36.9 ± 2.3
Oxalate	2.2 ± 1.3	70.8 ± 17.4	-41.4 ± 1.6	8.8 ± 3.0	-32.6 ± 1.6
Acetate	1.0 ± 0.2	83.6 ± 6.9	-28.8 ± 0.4	-5.3 ± 0.3	-34.1 ± 0.4
Citrate	2.4 ± 1.0	85.6 ± 3.4	-17.7 ± 1.1	-14.5 ± 1.7	-32.2 ± 1.2

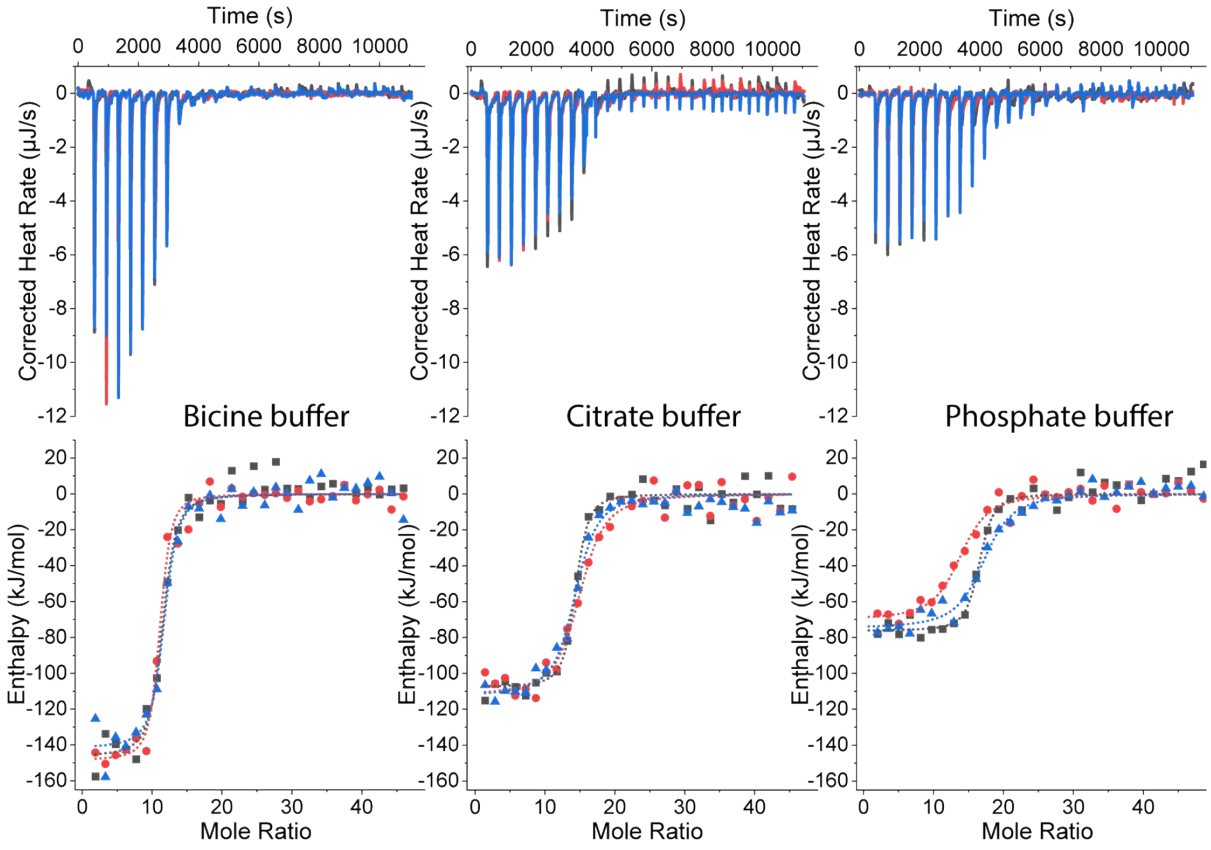


Fig. S2. ITC raw data (lines) and enthalpograms (dots) of alginate β-Lg coacervation with bicine buffer, citrate buffer or phosphate buffer, all at pH 2.65 (I = 20 mM). Dotted lines are fitted with an n equal, independent sites model. Each color indicate an individual measurement.

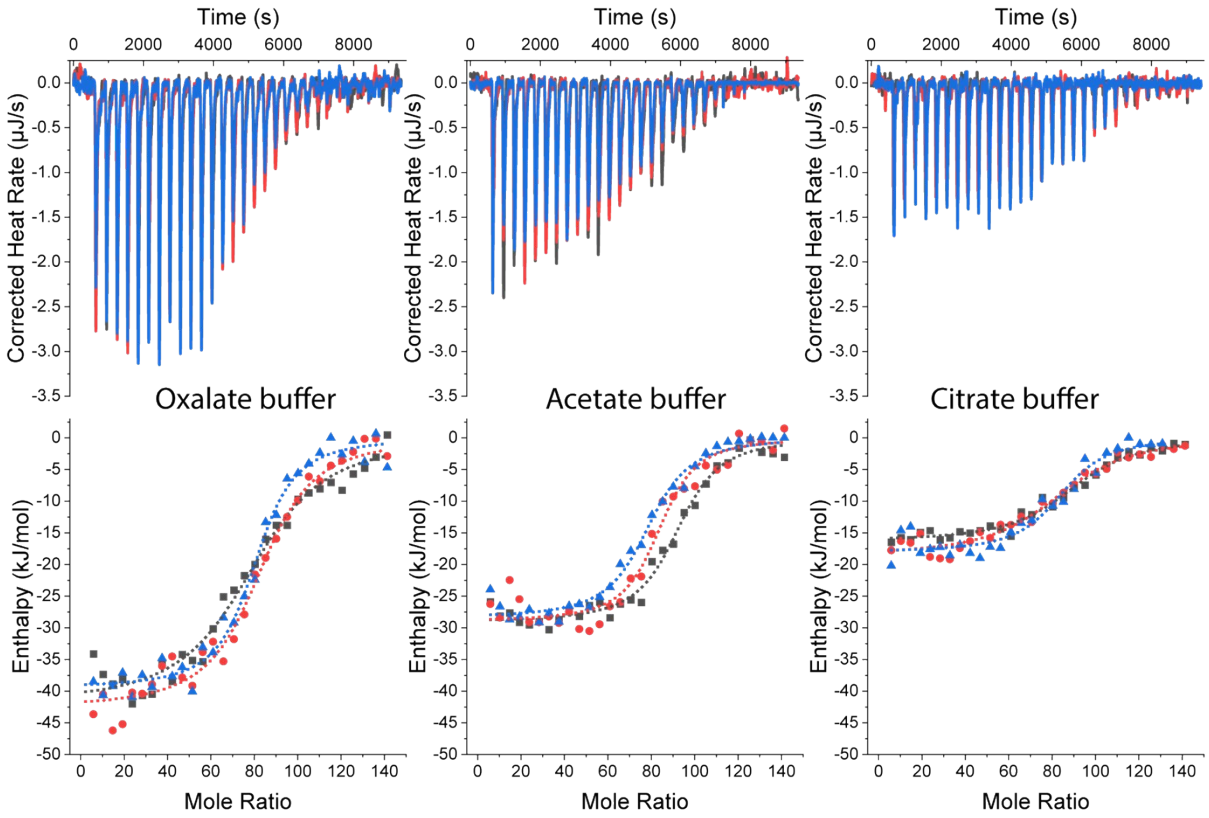


Fig. S3. ITC raw data (lines) and enthalpograms (dots) of alginate β-Lg coacervation with bicine buffer, citrate buffer or phosphate buffer, all at pH 4.0 (I = 20 mM). Dotted lines are fitted with an n equal, independent sites model. Each color indicate an individual measurement.

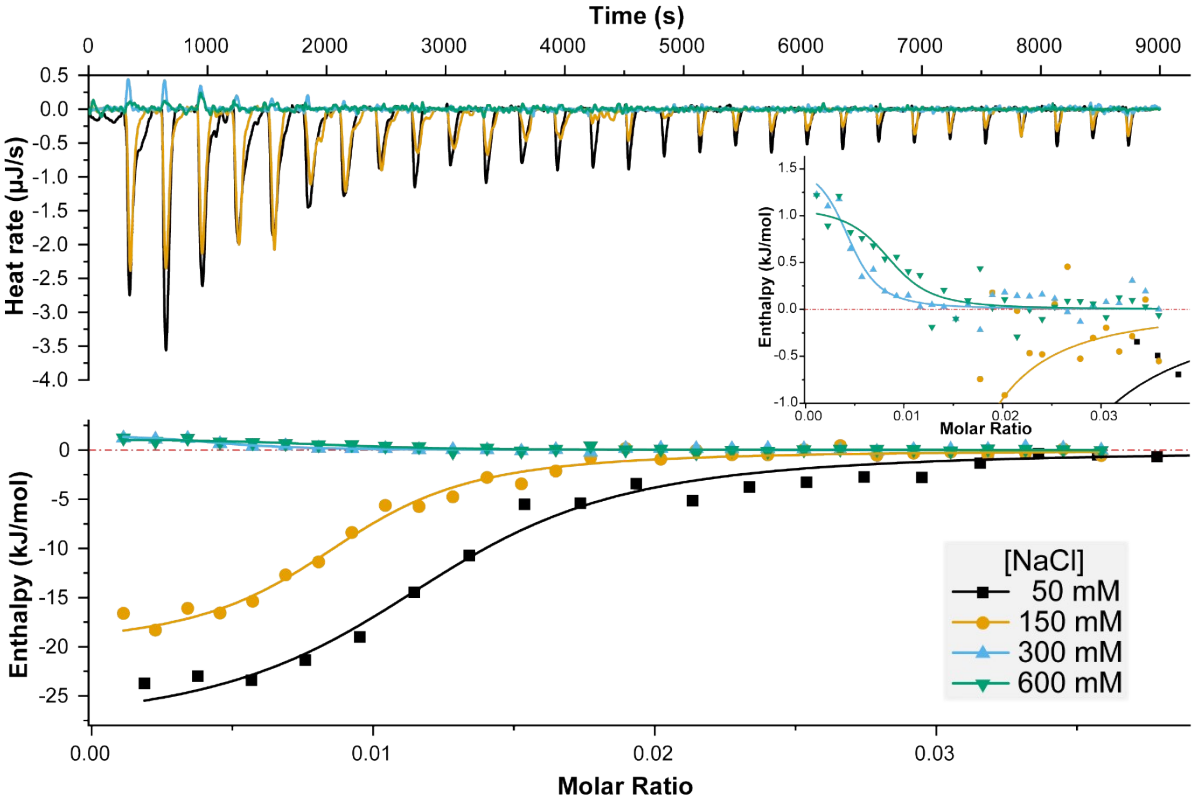


Fig. S4 Interactions of 4 µM AlgM (1.4 mg/mL) titrated into 27 µM β-Lg (0.5 mg/mL) measured by ITC at pH 4.0 with varying NaCl concentration of 50 mM (yellow), 150 mM (black), 300 mM (blue) and 600 mM (green). ITC raw data (top) of β-Lg titrated with AlgM and enthalpograms with model fits (bottom).

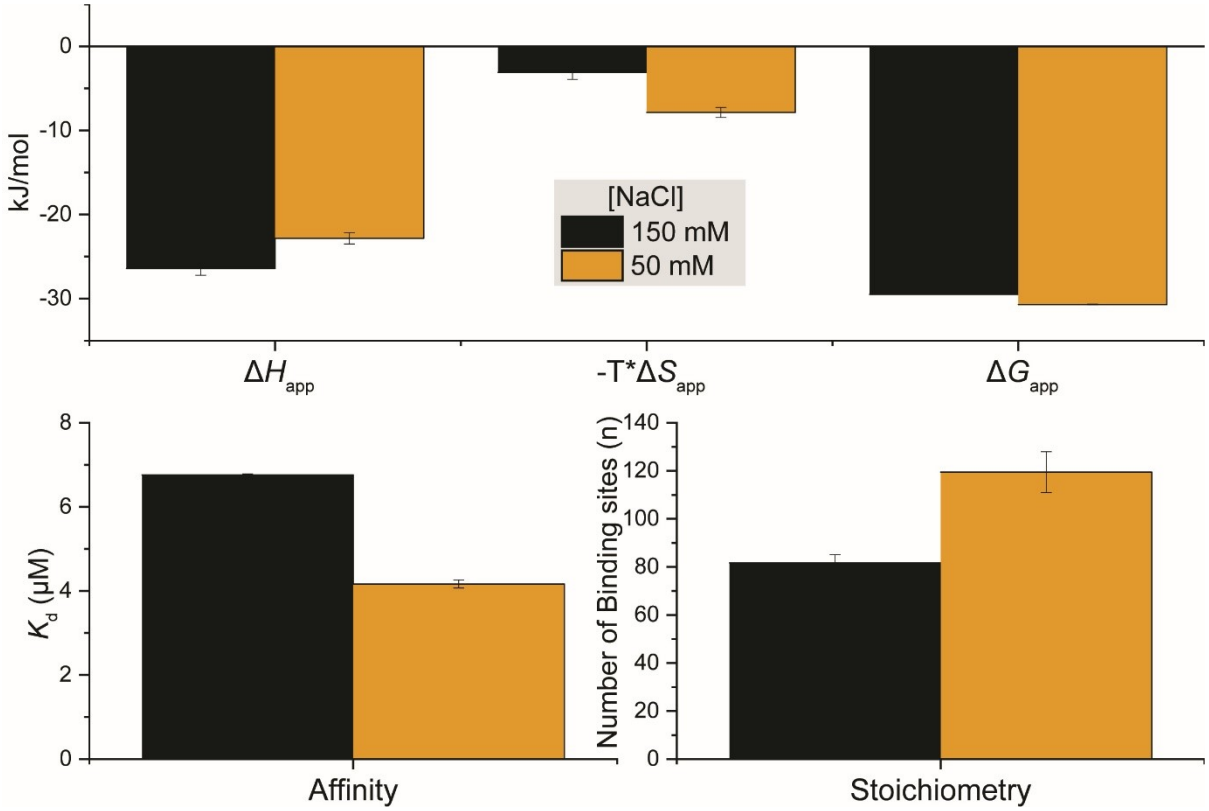


Fig. S5. Binding parameters obtained by fitting ITC thermograms (Fig. 3) with a one site independent binding model. All thermodynamic energies as well as affinities are given for β-Lg molecules, stoichiometry describes how many β-Lg molecules are bound by one alginate molecule.

Table S2. Binding parameters obtained by fitting ITC thermograms (Fig. S2) with a one site independent binding model (sample size = 3).

	50 mM NaCl	Std. dev.	150 mM NaCl	Std. dev.
K_d (M)	5.15E-06	0.02E-06	5.34E-06	0.03E-06
n	125.5	7.7	79.3	5.9
ΔH_{app} (kJ/mol)	−23.3	2.1	−25.8	2.9
−TΔ*S* (kJ/mol)	−6.9		−4.3	
Δ*G* (kJ/mol)	−30.2		−30.1	

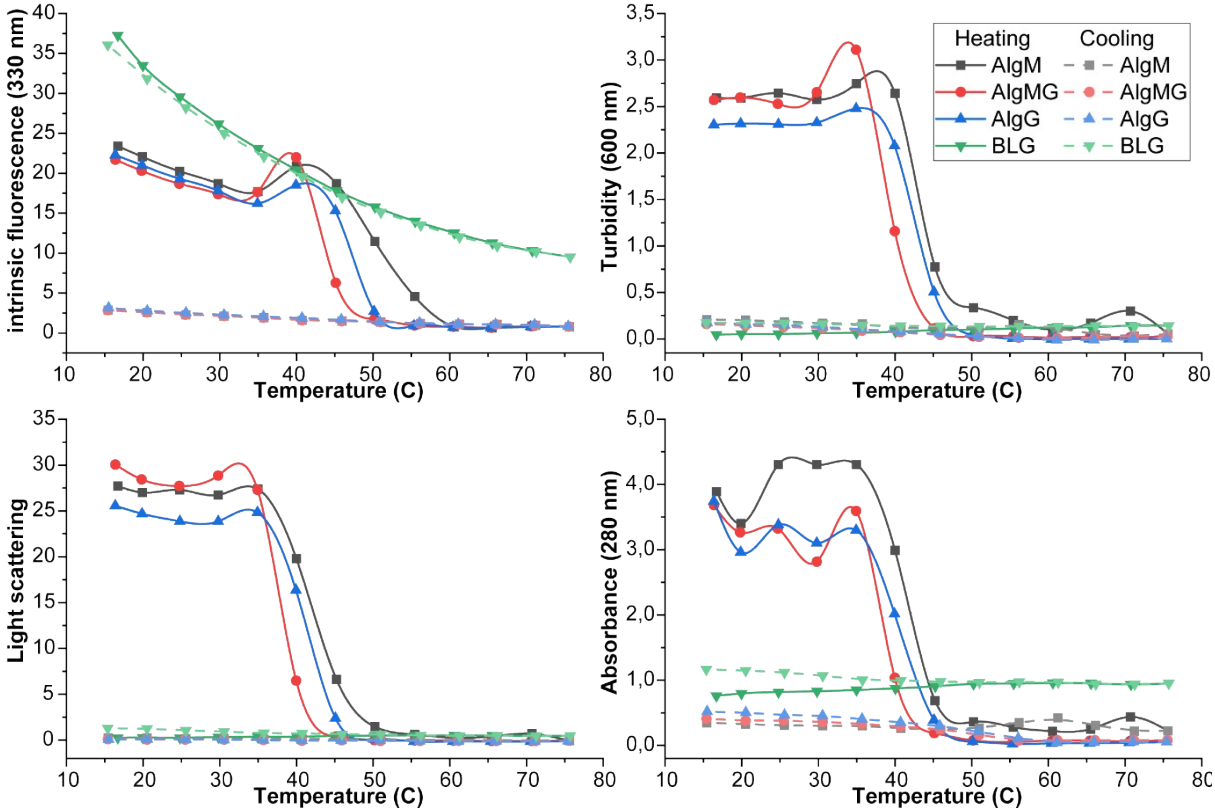


Fig. S6. The effect of heat on β-lactoglobulin alginate complexes was followed by Intrinsic fluorescence, turbidity, static light scattering and absorbance at 280 nm at pH 4.00. β-Lg (27 µM (0.5 mg/mL)), mixed with 0.67 µM alginate (0.2–0.3 mg/mL, AlgM (black), AlgMG (red) or AlgG (blue)) or without (green) was heated from 15 to 75°C (solid lines) and cooled (broken lines) in steps of 5°C.

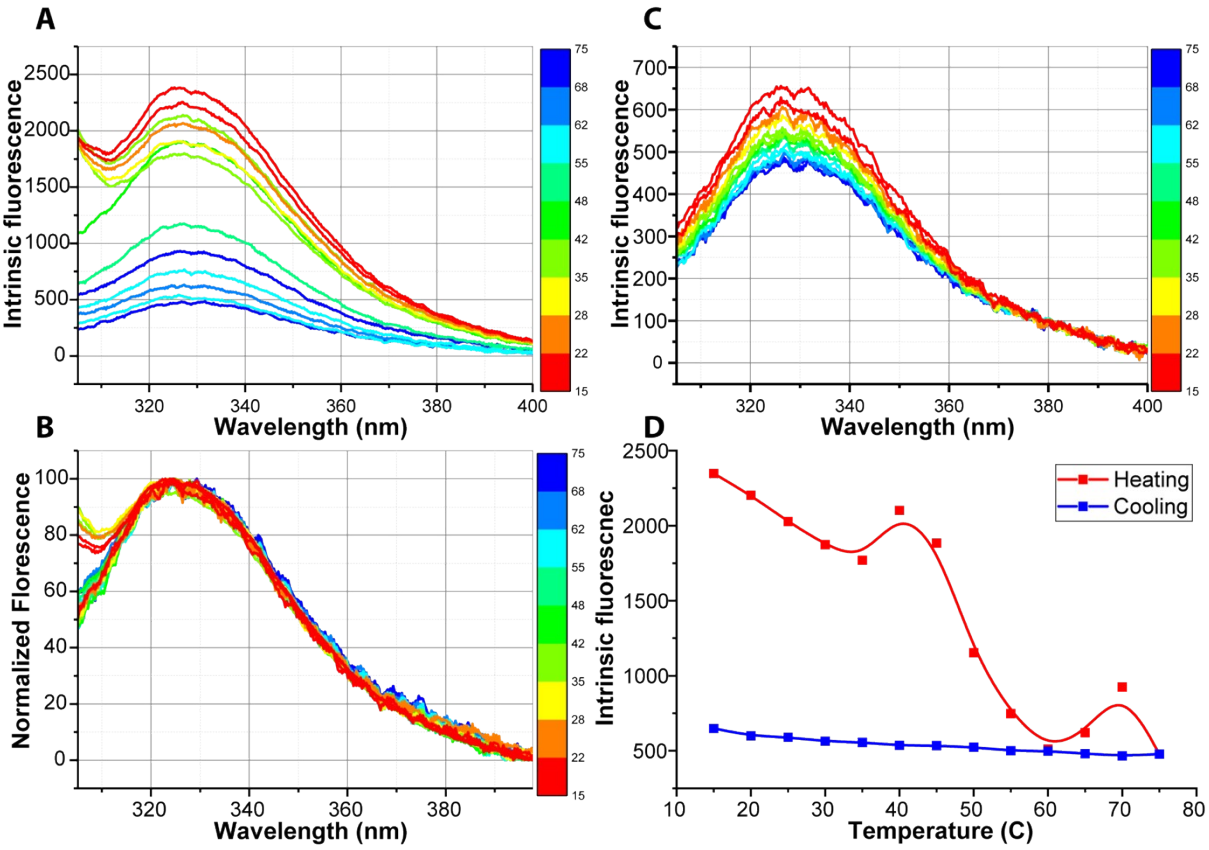


Fig. S7. Intrinsic fluorescence of β-Lg in complex with AlgM at pH 4.00, heated and cooled. A) Intrinsic fluorescence spectra during heating 15–75°C. B) Intrinsic fluorescence spectra during cooling 75–15°C. C) Normalized intrinsic fluorescence spectra during heating and cooling, overlayed. D) Intrinsic fluorescence at 330 nm plotted against increasing (red) and decreasing (blue) temperature.

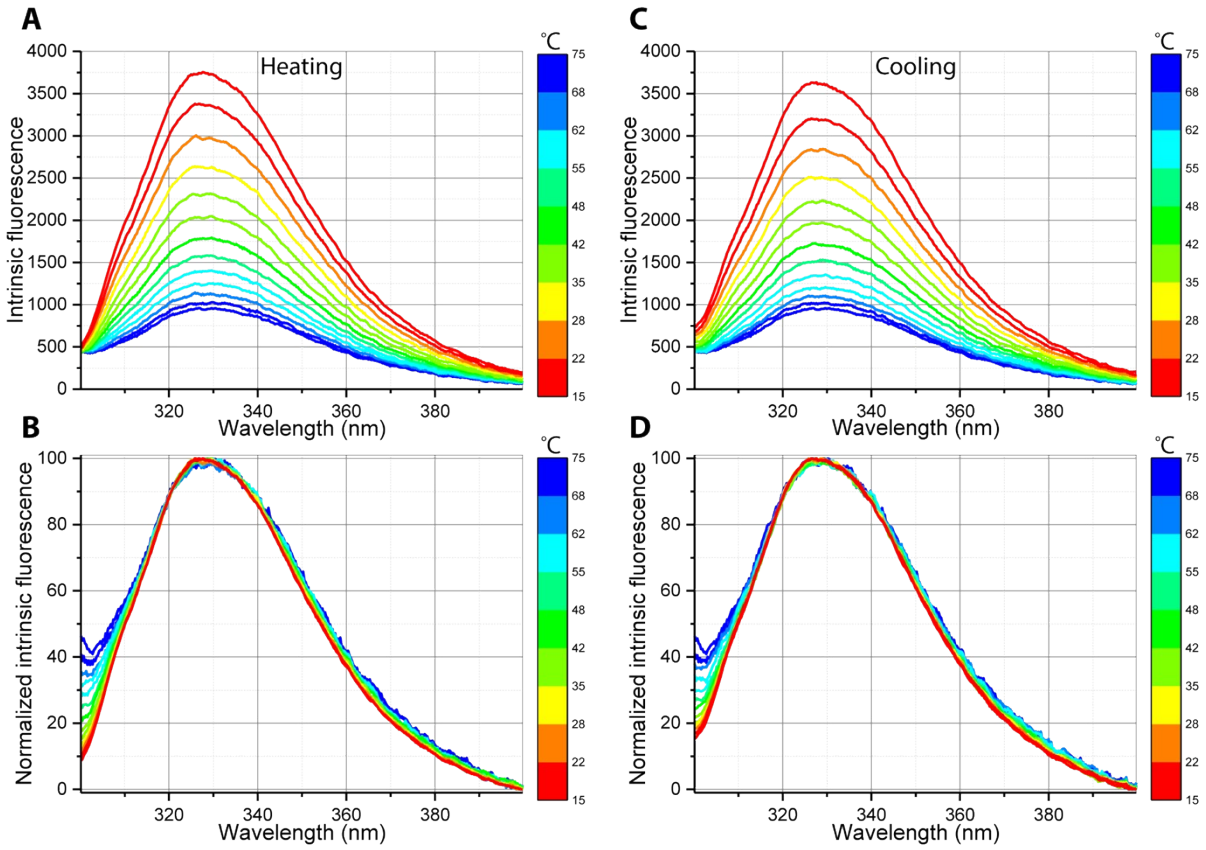


Fig. S8. Intrinsic fluorescence of free β-Lg at pH 4.00, heated and cooled. A) Intrinsic fluorescence spectra during heating 15–75°C. B) Intrinsic fluorescence spectra during cooling 75–15°C. C) Normalized intrinsic fluorescence spectra during heating. D) Normalized intrinsic fluorescence spectra during cooling.

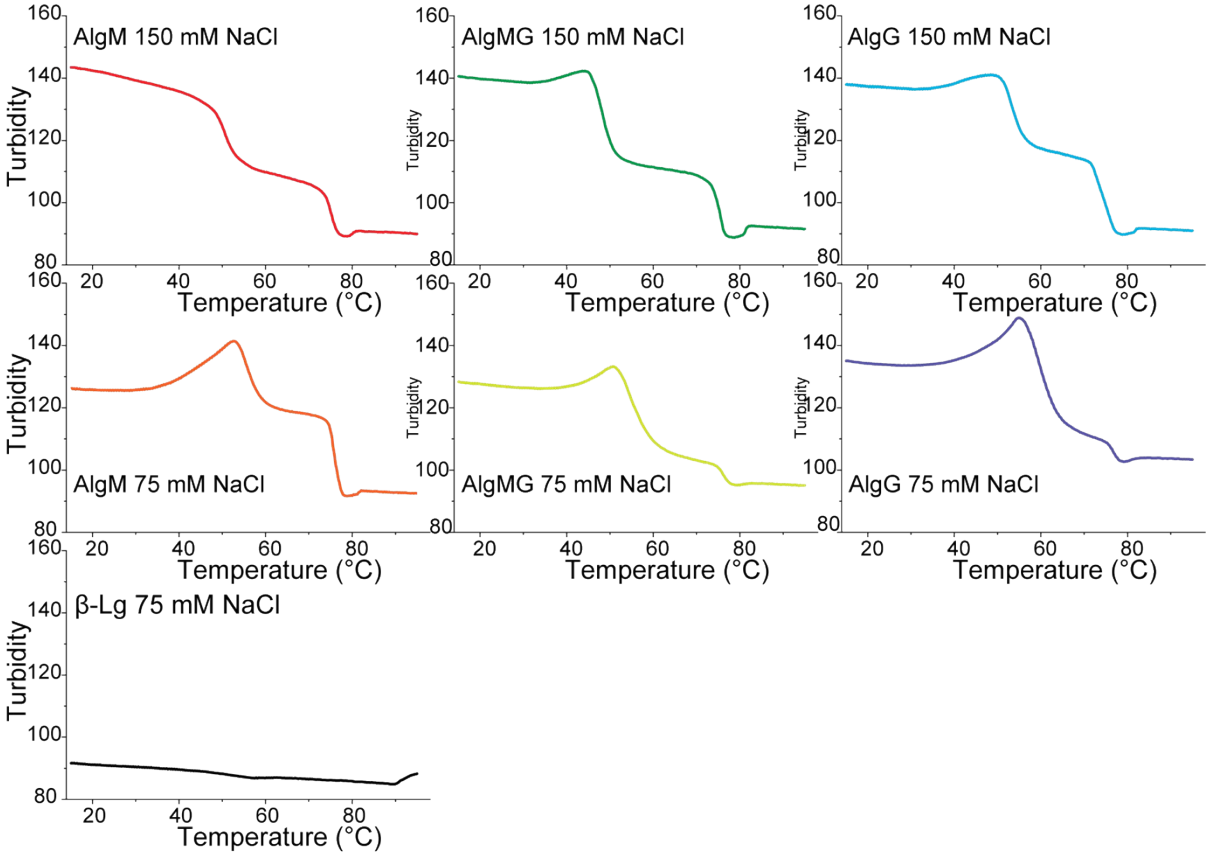


Fig. S9 Thermal stability of alginate β-Lg complexes at pH 4.00 measured by turbidity. β-Lg (27 µM, 0.5 mg/mL) mixed with 0.67 µM alginate (0.2–0.3 mg/mL at either 75 mM NaCl (orange, olive and purple) or 150 mM NaCl (red, green and cyan) and free 27 µM (0.5 mg/mL) β-Lg (black)).

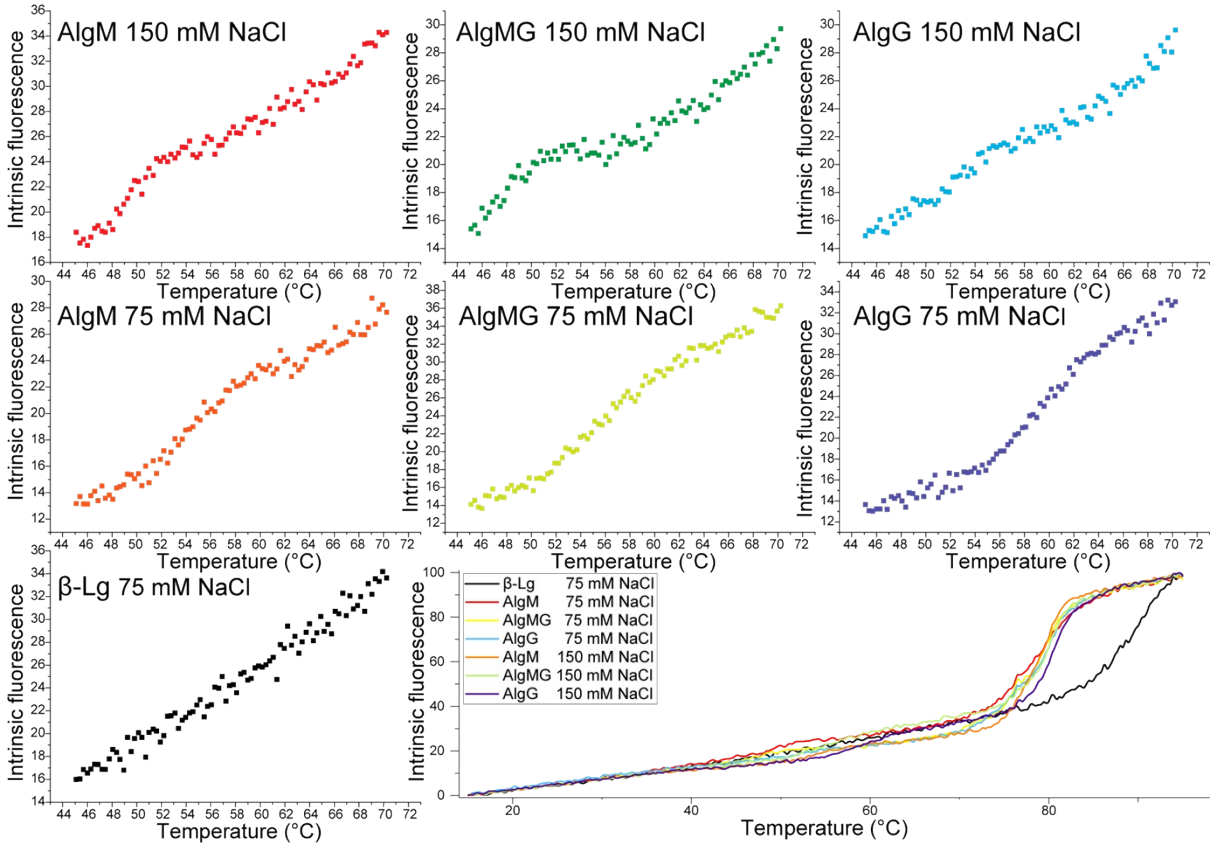


Fig. S10. Thermal stability of alginate β-Lg complexes at pH 4.00 measured by intrinsic fluorescence. β-Lg (27 µM, 0.5 mg/mL) mixed with 0.67 µM alginate (0.2–0.3 mg/mL at either 75 mM NaCl or 150 mM NaCl and free 27 µM (0.5 mg/mL) β-Lg. Scatter plots are individual zooms of the temperature range 42-72°C.

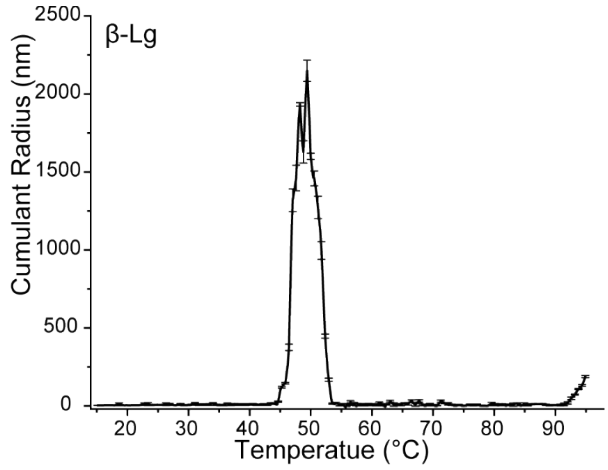


Fig. S11. Cumulant radius of 27 µM β-Lg (0.5 mg/mL) at increasing temperature.

Table S3. Melting temperatures determined by JBS Thermofluor fluorescence change (lowest points in the -d(RFU)dT Fig. S14). n.d. means that a melting temperature could not be determined within the temperature range (sample size = 3).

	75 mM NaCl		150 mM NaCl	
	Tm (°C)		Tm (°C)	
β-Lg	89.5	± 0.50	> 95.0*	± n.d.
AlgM	76.0	± 0.33	77.0	± 0.10
AlgMG	76.0	± 0.30	77.0	± 0.27
AlgG	76.0	± 0.55	77.5	± 0.33

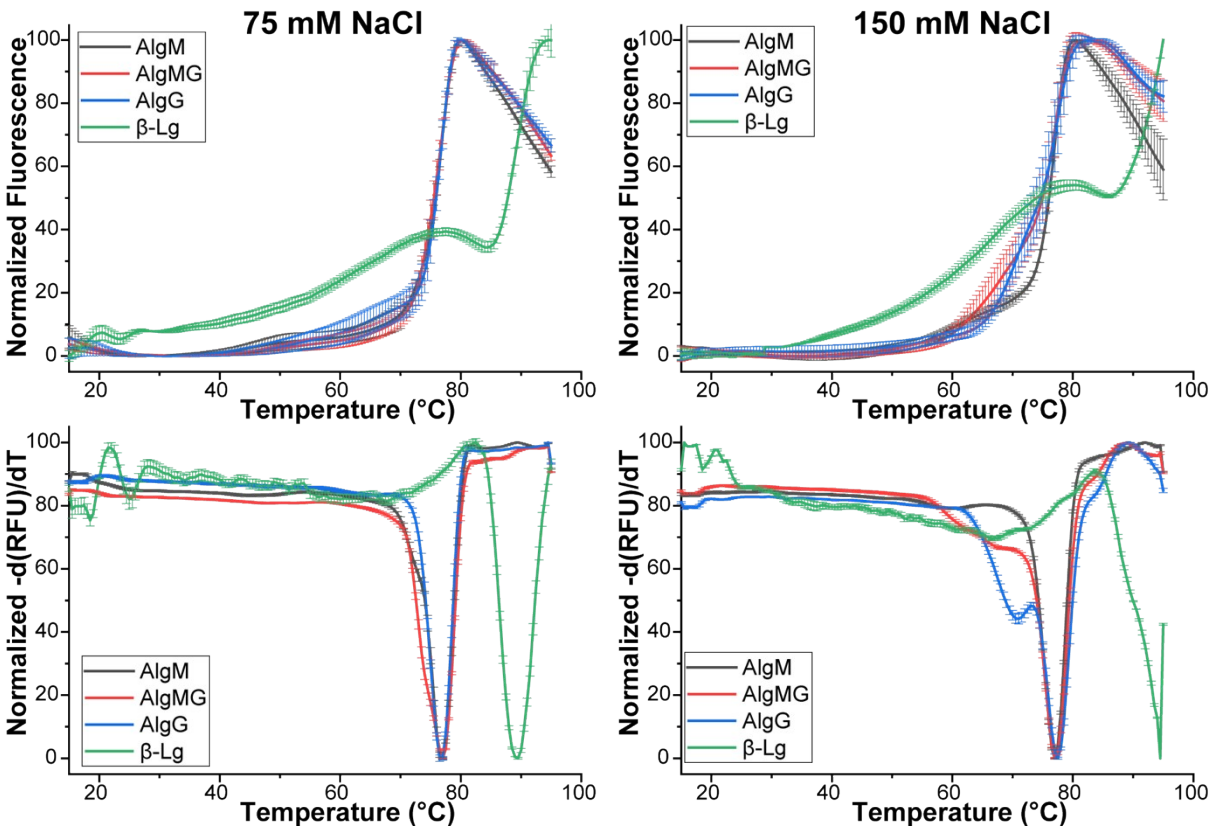
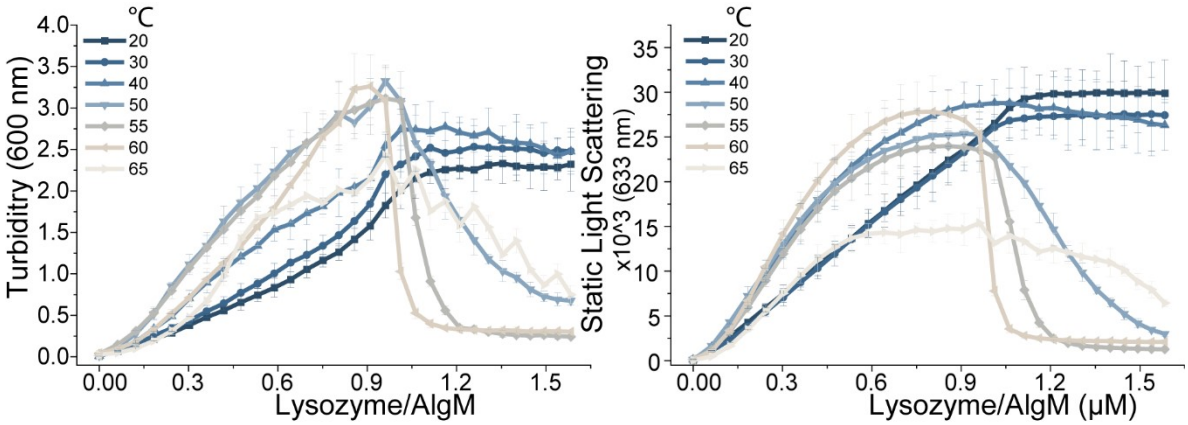


Fig. S12. Hydrophobicity changes at increasing temperature from 15 to 95°C, determined by JBS Thermofluor fluorescence. Top panels show normalized fluorescence (580 nm) and bottom panels show the first derivative (-d(RFU)/dT) of the top panels. Melting temperatures are determined as the peaks observed in the first derivative graphs. All samples were measured 6 independent times, lines show the mean data and error bars are the resulting standard errors of the means.



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101 **Fig. S13** Effect of increasing temperature on alginate lysozyme coacervation at pH 7.0. A) Turbidity
102 measured at 600 nm for coacervation from 20 to 65°C. B) Static light scattering measured at 633 nm
103 for coacervation from 20 to 65°C.

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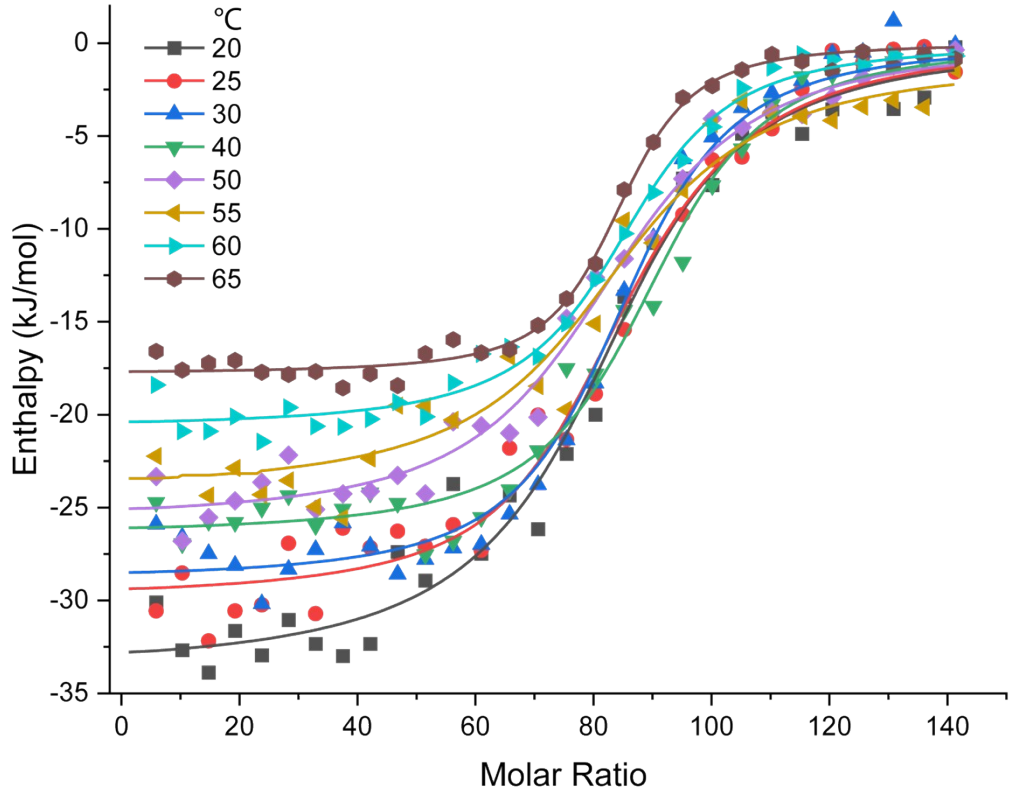

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106 **Fig. S14.** Averaged enthalpograms (dots) of alginate β-Lg coacervation at 20, 25, 30, 40, 50, 55, 60
107 and 65°C. Lines are the averaged fitted n equal, independent sites model for each temperature. Non-
108 averaged data are found in Figs. S14 and S15. Molar ratio describes n β-Lg /n alginate.

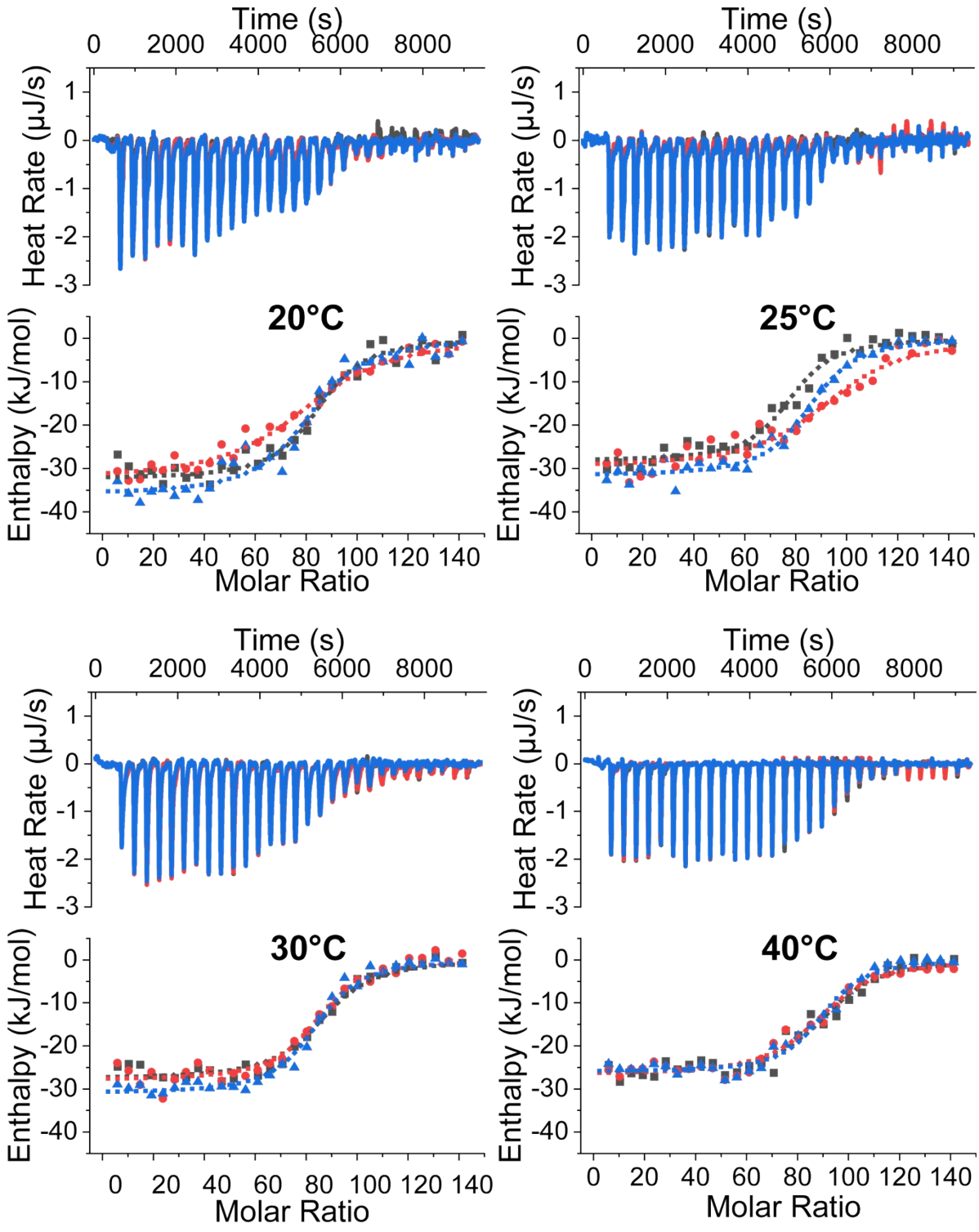


Fig. S15. ITC raw data (lines) and enthalpograms (dots) of alginate β-Lg coacervation at 20, 25, 30 and 40°C. Dotted lines are fitted with an n equal, independent sites model. Molar ratio describes n β-Lg /n alginate

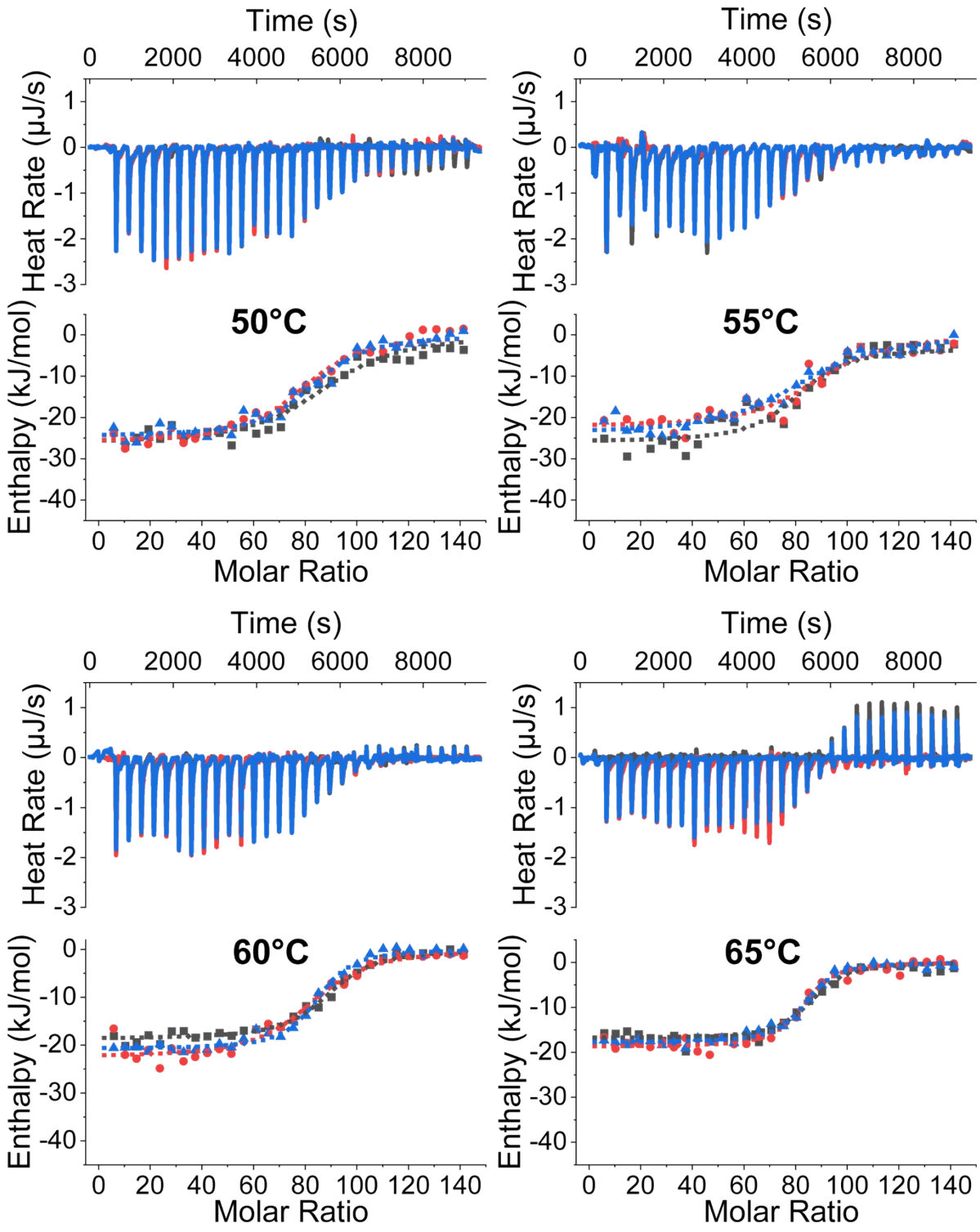


Fig. S16. ITC raw data (lines) and enthalpograms (dots) of alginate β-Lg coacervation at 50, 55, 60 and 65°C. Dotted lines are fitted with an n equal, independent sites model. Molar ratio describes n β-Lg /n alginate.

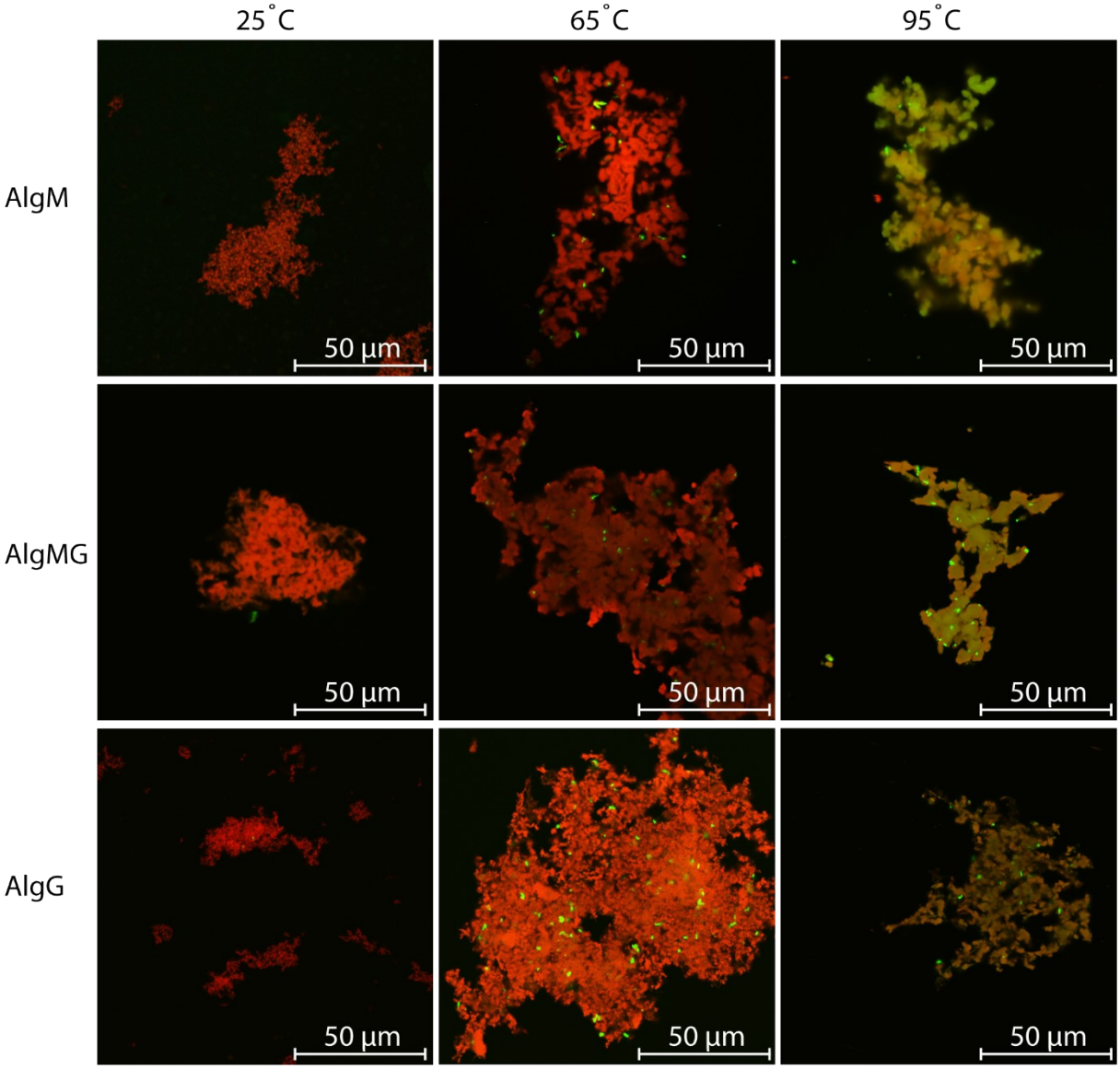
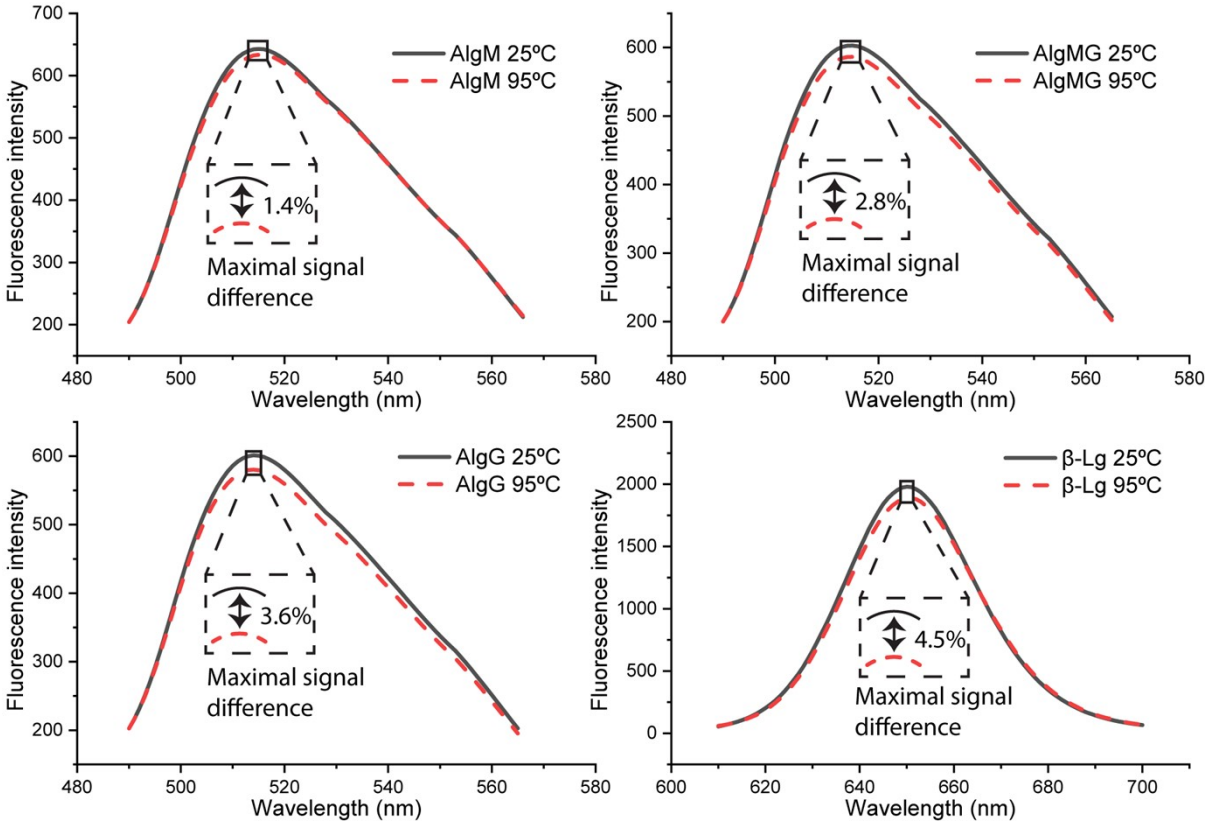


Fig. S17 Images of fluorescein labelled alginate in complex with Abberior STAR RED labelled β-Lg at pH 4.0, *I* = 15 mM, heat treated at 25, 65 and 95°C for 5 min. Images were aquired with 100x immersion oil objective where complexes were excited at 488 and 630 nm.



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123 **Fig. S18.** Fluorescence spectra of AlgM, AlgMG, AlgG and β-Lg, at 25°C or heated to 95°C.

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