

Supplementary Information:

Choline dendrimers as generic scaffolds for the non-covalent synthesis of multivalent protein assemblies.

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S1: Materials

Solvents were obtained from Biosolve and were of p.a. quality. Di (N-succinimidyl) carbonate (Fluka), dialysis tubing (Spectrum Labs), and choline chloride (> 99%, Fluka) were used as received. Poly(propylene imine) dendrimers were obtained from SyMO-Chem BV (Eindhoven, The Netherlands). Rat tail collagen type I (C7661) and human collagen type IV (C7521) were obtained from Sigma. Acetonitrile was stored on 4 Å activated molecular sieves (Merck) under an argon gas atmosphere. Triethylamine (>99%, Fluka) was stored on potassium hydroxide pellets (Merck). Ion exchange resin DOWEX 1X8-50 (Cl⁻ form) was obtained from Acros. Prior to use, a column was first washed with demineralised water, followed by washing with methanol. A methanolic solution of the dendrimer was applied to the column and eluted with methanol. Weakly basic ion exchange resin Amberlite IRA-95 (Aldrich) was washed with water, methanol, and again water before use. PD-10 columns (GE Healthcare) were washed with water according to the manual. Reactions were performed under a dry argon gas atmosphere. **g5-cho** dendrimers were synthesized as previously described¹

S2: Synthesis of g5-cho/FITC dendrimer

DAB-dendr-(NH₂)₆₄ (159 mg; 0.02218 mmol) was dissolved in 8 mL of water. FITC (11 mg; 0.0282 mmol) was stirred with 1 mL of methanol and 20 µL triethylamine for one minute, resulting in a clear solution. This solution was added portion wise during 1 min to the dendrimer solution. After stirring for 27 h at room temperature, 0.40 mL of triethylamine and 8 mL of THF was added. During 1 min, 478 mg of choline NHS carbonate¹ was portion wise added. After 3.5 hours, an additional amount of 77 mg choline NHS carbonate was added. After stirring for 16 h, the clear mixture was evaporated. Then it was dissolved in 6 mL of water and filtered (5

micrometer filter) to remove some precipitate. 0.50 mL of triethylamine was added, and the mixture was purified by PD-10 column filtration. After eluting over a Cl⁻ anion exchange column, and freeze drying from water, 230 mg of product was obtained.

¹H-NMR (D₂O; 400 MHz): δ 7.0-6.90 + 6.45-6.35 (weak signals, aromatic fluorescein protons), 4.45-4.05 (m, 128H), 3.70-3.50 (m, 128H), 3.20-2.80 (m, 704H), 2.55-2.15 (m, 372H), 1.65-1.25 (m, 246H).

¹³C NMR (D₂O, 100 MHz): δ 156.8, 64.8, 58.4, 53.7, 52.7, 51.7, 51.5, 50.2, 38.9, 25.4, 24.8, 21.9.

S3: Construction of the C-LytCNA35 and C-LytCNA35-Y175K fusion proteins

pQE30CNA35, which includes the residues 31 to 344 of *S. aureus* CNA protein inserted into the multiple cloning site (MCS) of the pQE30 plasmid (Qiagen GmbH)², was generously donated by Magnus Höök (Texas A&M University, USA). A DNA fragment containing CNA35 (CNA₃₁₋₃₄₄) nucleotide sequence was PCR amplified from pQE30CNA35 plasmid using oligonucleotides C35FW (CCATCACGCATCCCACGAGATATTTC) and C35REV (ACCACCCAGCTGCTAATTAAGCTTGGCTGCAGG), introducing flanking restrictions sites for BamHI and PvuII. The PCR products were purified, digested with these enzymes and ligated into the pALEX2 expression vector (Biomedal, S.L.). The ligation was carried out using T4 DNA ligase (Roche Diagnostics GmbH), as described by the manufacturer. The ligation mixture was subsequently digested with KpnI, whose unique restriction site in pALEX2 is in the region cleaved out by BamHI and PvuII, to eliminate non-digested pALEX2 molecules. Finally XL-1 Blue cells were transformed with the ligation mixture and plated on LB-Agar plates supplemented with ampicillin. Single colonies were selected and their plasmid DNA was sequenced to check the correct insertion of the CNA35 sequence leading to the pCLYTCNA35 plasmid. pCLYTCNA35 was then subjected to directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, #200519) with the complementary oligonucleotides CAN-YKL (TTCTATAAGAAACGGGAGATATGCTA) and CAN-YKR (TTTCTTATAGAAAACACTACTTGTTC),³ to produce pCLYTCNA35YK plasmid, coding for the C-LytCNA35-Y175K protein.

S4: Expression and purification of C-LytA, CNA35, C-LytGFP, C-LytCNA35 and C-LytCNA35-Y175K

C-LytA was purified by affinity chromatography from the overproducing *E. coli* strain RB791 [pCE17] as previously described.⁴ C-LytGFP was expressed and purified as previously described.¹ C-LytA/CNA fusion proteins expression was achieved from the *E. coli* REG1 strain, transformed with the corresponding pALEX derivative. Purification was carried out using the LYTAG Purification System (Biomedal, S.L.) as described by the manufacturer. CNA35 was purified from *E. coli* strain BL21(DE3) [pQE30CNA35] as previously described⁵. All proteins were extensively dialyzed after purification against 10 mM NH_4HCO_3 at pH 7 and freeze-dried for 24 h at -80 °C for storage.

S5: Dynamic light scattering

Experiments were performed on a Protein Solutions DynaPro MS/X apparatus equipped with an 824.7-nm-wavelength laser. Samples were equilibrated in TBS (10 mM Tris-HCl at pH 7.4 with 150 mM NaCl) and filtered twice with a 0.1 μm Anotop syringe filter (Whatman) prior to measurement. Autocorrelation curves were analyzed with Dynamics V6 software (Wyatt Technology Corp.) which uses the regularization method to obtain a particle distribution.

S6: Sedimentation velocity

The experiments were carried out at 48,000 rpm and 20 °C in a XL-I analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with an UV-vis and IF detection system, an An-50 Ti rotor, and 12 mm double-sector centrepieces. Sedimentation profiles were registered using the IF detection system and additionally at 495 nm for fluorescein-labelled samples. The sedimentation coefficient distributions were calculated by least-squares boundary modelling of sedimentation velocity data using $c(s)$ method as implemented by the SEDFIT program.⁶ These s -values were corrected to standard conditions (water, 20°C, and infinite dilution) to obtain the corresponding standard s -values ($s_{20,w}$) using SEDNTERP program.⁷ Prior to measurements, samples were extensively dialyzed in TBS.

S7: Solid phase binding assays

96-well Sarstedt polystyrene transparent microplates (Sarstedt, 82.1581) were coated overnight at 4 °C with 3.2 µg/well (50 µL) rat tail collagen type I or human collagen type IV in TBS. After overnight incubation, the plates were blocked with 100 µL TBS containing 5% (w/v) skim milk powder for 2 h at room temperature. After washing the plates 3 times with 300 µL TBS, the plates were incubated with the indicated combination of proteins and/or choline dendrimers in TBS supplemented with 1% BSA for 3 h at room temperature. Prior to their addition to the wells, samples were centrifuged 5 minutes at 10,000 rpm in a tabletop centrifuge, adding only the supernatant. When indicated 0.5 M choline or 0.5 M NaCl were added. Upon incubation, plates were washed 5 times with 10 mM Tris-HCl, 500 mM NaCl, pH 7.4 and subsequently washed 2 times with TBS. The fluorescence of the fluorescein-labelled choline dendrimers was measured at 520 nm in triplicate on a BMG Fluorstar Galaxy plate reader after excitation at 480 nm. In all experiments, every condition was tested in parallel in at least 3 wells, and the data for the three wells were then averaged.

S8: Surface Plasmon Resonance

Measurements were performed on a Biacore T100. Buffers were freshly prepared and 0.22 µm-filtered before use. Rat tail collagen type I (Sigma, C7761) was covalently immobilized on a Biacore CM5 chip using the Amine Coupling kit (Biacore Inc., BR-1000-50) according to manufacturer's instructions. In all the experiments, samples were flown first through a reference flow cell with no collagen immobilized. The carboxyl groups in the reference channel were blocked with ethanol amine groups by performing a standard amine coupling reaction but flowing only 1 M ethanolamine after surface activation. A 10 mM glycine-HCl buffer at pH 1.5 was used for regeneration of the collagen surface at the end of each cycle. Titrations with proteins in the absence of dendrimers were performed using either HBS-EP+ buffer (10 mM Hepes at pH 7.4 with 10 mM EDTA, 150 mM NaCl and 0.05% (w/v) Surfactant P20) or 100 mM PBS at pH 7 with 500 mM NaCl, as indicated. Titrations with proteins in the presence of dendrimers were always performed with the latter buffer. Three buffer injections were performed before the start of each titration. In every titration, at least one of the concentrations was injected in duplicate. Binding curves were obtained from titrations

by plotting the response level at equilibrium (flat response level after injection) against the protein concentration injected.

S9: Analysis of Surface Plasmon Resonance data

Apparent affinities were obtained from binding curves by fitting them using either a model for one binding site (equation 1) or a model for two binding sites (equation 2), as indicated:

$$(1) \quad R = \frac{R_{MAX} \cdot C}{K_D + C}$$

$$(2) \quad R = \frac{R_{MAX1} \cdot C}{K_{D1} + C} + \frac{R_{MAX2} \cdot C}{K_{D2} + C}$$

where R is the number of response units when the binding arrives at the equilibrium and C is the analyte concentration in both equations. In equation 1, R_{MAX} is the maximum binding response and K_d represents the dissociation constant of the reaction. In equation 2, R_{MAX1} and R_{MAX2} are the maximum responses for site 1 and site 2, respectively, K_{d1} and K_{d2} are dissociation constants for site 1 and site 2 respectively.

S10: Tissue samples preparation and visualization

Frozen porcine pericardium was defrosted and cut into 1 x 1 cm pieces. After washing and blocking with 3 % milk powder in 10 mM Tris-HCl with 0.5 M NaCl at pH 7.4 for 1 h, tissue slices were incubated 0.5 μ M **g5-cho/FITC** in the absence or presence 5 μ M C-LytCNA35, C-LytCNA35-Y175K, or C-lytA in 10 mM Tris-HCl with 0.5 M NaCl at pH 7.4 for 2 h at room temperature in the dark on a rocking platform. As a positive control, tissue samples were also incubated with 0.5 μ M CNA35 covalently labeled with Oregon Green 488 as previously described.⁵ Subsequently, the tissue samples were 1 time washed with 10 mM Tris-HCl with 0.5 M NaCl at pH 7.4 for 15 minutes. Porcine pericardium was imaged using an inverted Zeiss Axiovert 200 microscope coupled to an LSM 510 Meta (Carl Zeiss, Germany) confocal laser scanning microscope with a 20x objective. An argon laser at 488 nm was used for excitation and a band pass filter of 500-550 nm was used to detect fluorescence emission. Microscopy settings were kept identical for all samples.

References

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Figure S1

Dynamic light scattering (DLS) characterization of C-LytCNA35 complexes with g5-cho. **(A)** Autocorrelation profiles of 10 μM C-LytCNA35 either alone (black line) or in the presence of 1 μM (green line) or 5 μM (red line) **g5-cho**. The autocorrelation profile of 5 μM **g5-cho** is also shown for comparison (blue line). **(B)** Particle distributions obtained from autocorrelations profiles in A calculated with the regularization method used by Dynamics software (Wyatt Technology Corp.).

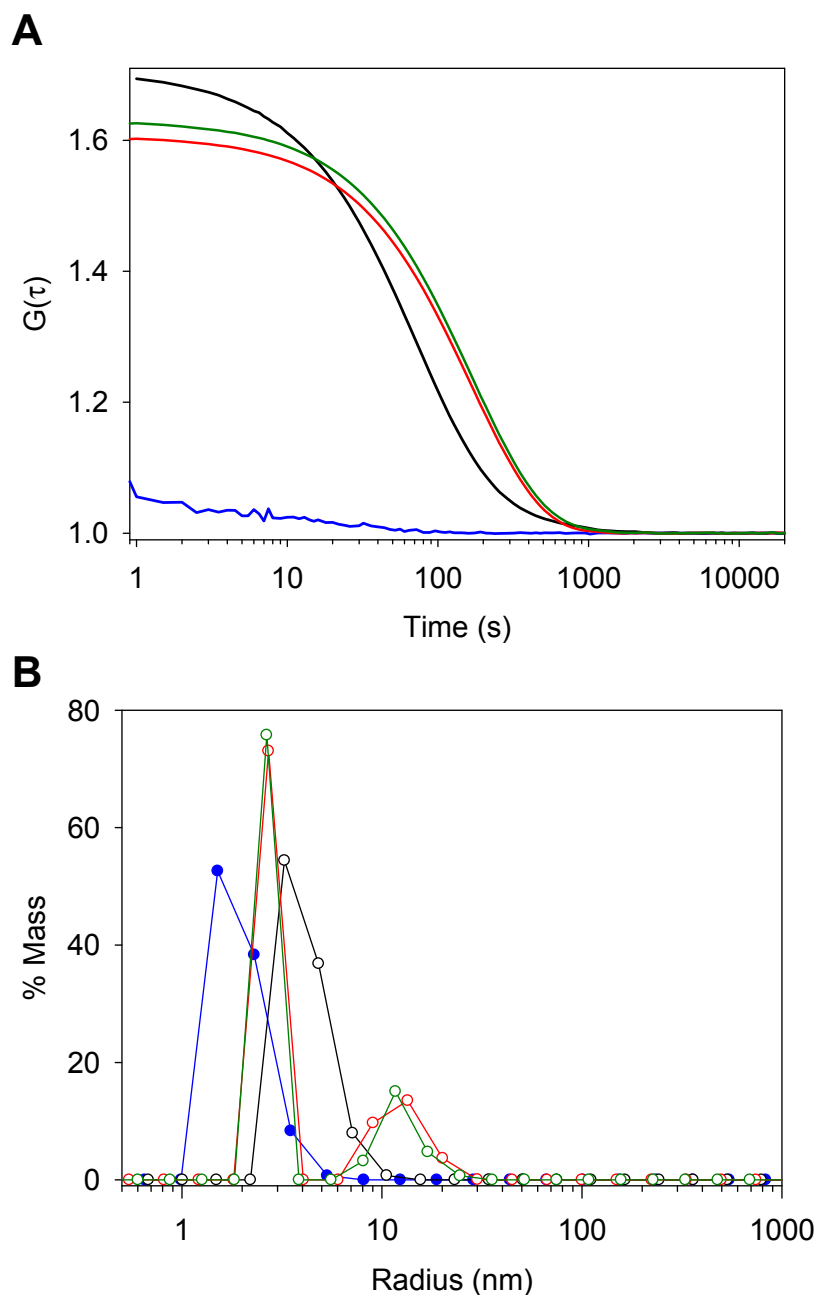


Figure S2

Characterization of C-LytCNA35 complexes with g5-cho/FITC by sedimentation velocity. (A) Sedimentation coefficient distribution of 5 μM C-LytCNA35 in the presence (dashed line) or absence of 0.5 μM g5-cho/FITC (straight line). Samples were monitored by interference. (B) Sedimentation coefficient distribution of 5 μM g5-cho/FITC in the presence (dashed line) or absence of 10 μM C-LytCNA35 (straight line). Samples were monitored by following absorbance of FITC at 495 nm.

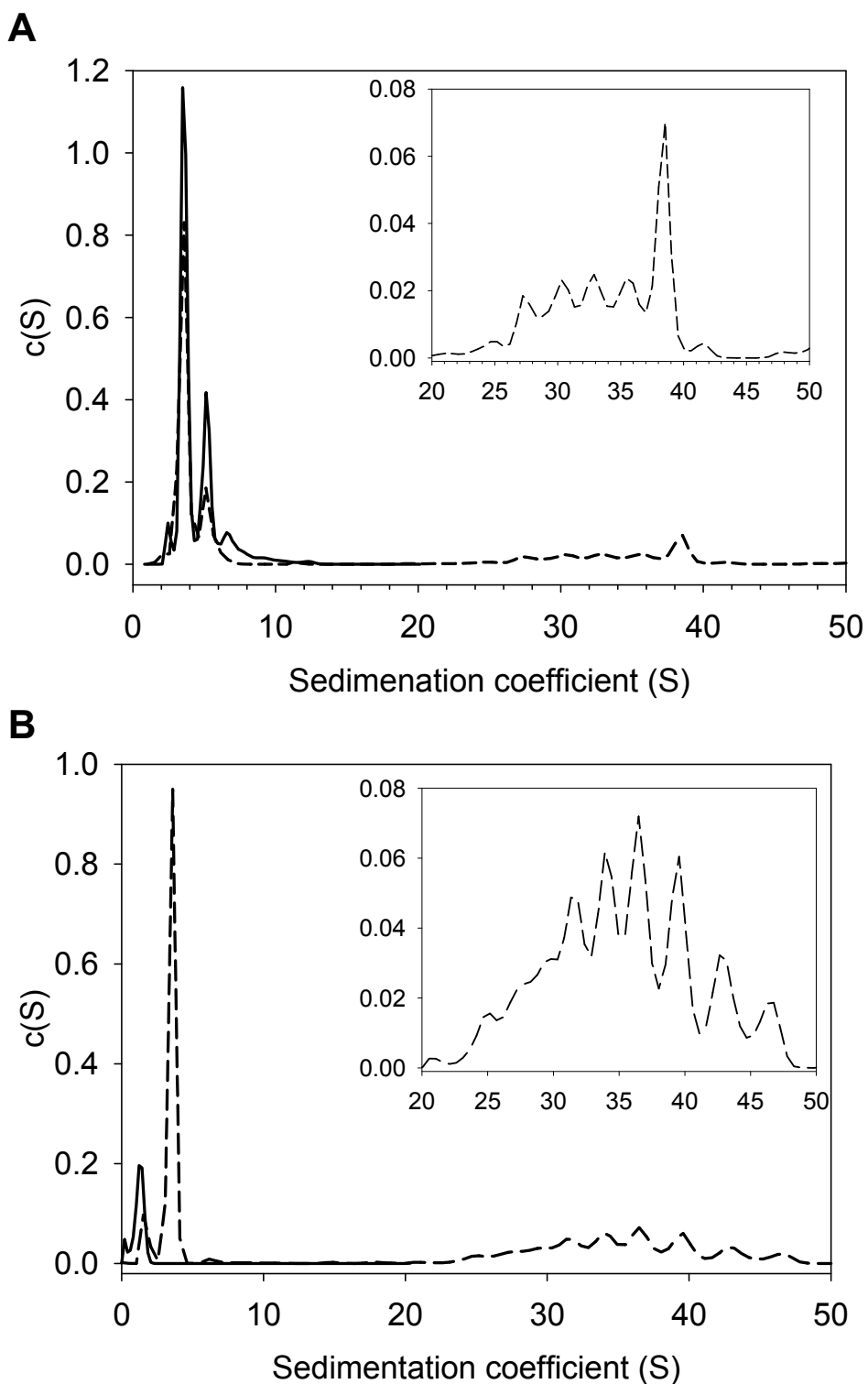


Table S1

Radiuses and molecular masses calculated with the regularization method analysis of autocorrelation profiles of Supplementary Figure 1 A. Masses and radiuses were calculated assuming a Rayleigh Spheres model.

Sample	R1 (nm)	Mass1 (kDa)	R2 (nm)	Mass2 (kDa)
5 μ M g5-cho	2	43		
10 μ M C-LytCNA35	4.2	96		
10 μ M C-LytCNA35 + 1 μ M g5-cho	2.7	33	12.6	1300
10 μ M C-LytCNA35 + 5 μ M g5-cho	2.7	35	12.8	1300