Appendix 1. Ultracentrifugation analysis of TLR3ecd binding to dsRNA

Sample preparation: All samples were prepared in PiBS (pH 5.5) and were analyzed at 20.0°C using a Beckman Optima XL-A analytical centrifuge.

Sedimentation equilibrium methods: Samples (loading volume of 130 μL) were loaded in 6-channel epon charcoal centerpiece cells at A_{280} of approximately 0.25, 0.5 and 1.0, and studied at rotor speeds of 6 to 21 krpm. Data were acquired as an average of 4 absorbance measurements at 280 nm using a radial spacing of 0.001 cm. Sedimentation equilibrium was achieved within 42 hours. Data collected at different speeds and different loading concentrations were analyzed globally in terms of various species analysis models using SEDPHAT 4.3 software (1) to obtain the buoyant molecular mass. Solution densities ρ were measured at 20.00°C on a Mettler-Toledo DE51 density meter. The partial specific volume ν_{prot} for the protein portion of TLR3ecd was calculated based on the amino acid composition in SEDNTERP 1.1 software (2).

Sedimentation velocity methods: Samples (loading volume of 350 μL) were studied at rotor speeds of 45 or 50 krpm using aluminum double sector cells with data acquired as single absorbance measurements at 260 or 280 nm using a radial spacing of 0.003 cm. 90 – 100 scans were collected at approximately 3 minute intervals. Samples of the TLR3ecd were studied at a loading A_{280} of 0.8, whereas samples of the free dsRNA and various TLR3ecd:RNA mixtures were studied at loading A_{260} of around 1.0. Data were analyzed in SEDFIT 9.4, as well as SEDFIT β2d, in terms of a continuous c(s) distribution (3). Continuous c(s) distributions covered a sedimentation coefficient range of 2 – 16 S with a resolution of 100 and a confidence level (F-ratio) of 0.95. Excellent fits were obtained with rmsd ranging from 0.0050 to 0.0068. The experimentally determined solution density (ρ) was used for the calculations and a viscosity (η) of 0.0102 P was assumed. Data for the dsRNA and TLR3ecd alone were also analyzed in terms of a single non-interacting discrete species with excellent fits. Note that the sedimentation coefficients *s* reported are not corrected to $s_{20,w}$.

48 bp dsRNA and TLR3ecd are monodisperse monomers: Sedimentation equilibrium experiments were used to determine the mass and homogeneity of pure dsRNA and pure TLR3ecd. In each case, a global data analysis (using multiple loading concentrations and rotor speeds) returned excellent fits when either dsRNA or TLR3ecd was modeled as a single ideal solute (Figs. A1.1 and A1.2). For the dsRNA, an experimental buoyant mass of 15.5 ± 0.12 kDa was measured. In order to convert this value into an actual (experimental) molecular mass, a partial specific volume of 0.50 cm^3g^{-1} was assumed (partial specific volumes for nucleic acids vary from 0.50 to 0.55 cm^3g^{-1} depending on the solvent composition (4, 5)). This yielded a molecular mass of 31.2 ± 0.25 kDa (predicted mass = 31.1 kDa). The TLR3ecd was determined to have an experimental buoyant mass of 24.5 ± 0.2 kDa, a value that is larger than that calculated for the TLR3ecd based on amino acid composition alone, which is consistent with the fact that the protein is glycosylated. Assuming a partial specific volume of 0.65 cm^3g^{-1} for the carbohydrate moiety and assuming that buoyant molecular masses are additive (6), a molecular mass of 93.0 ± 0.6 kDa was calculated, with a carbohydrate contribution of 8.7 ± 0.6 kDa and a

partial specific volume of 0.7316 cm^3g^{-1} for the entire glycoprotein. As described in the main text, this molecular mass is consistent with previous observations (7). Sedimentation velocity profiling further confirmed the monodispersity of these samples (see Fig. 3D in the main text) and was used to estimate molecular masses of 32.1 ± 0.2 kDa and 91.5 ± 0.2 kDa for the dsRNA and TLR3ecd, respectively (assuming a viscosity of 0.0102 P and the partial specific volumes described above). Thus, both pure dsRNA and pure TLR3ecd are monomeric and monodisperse.

TLR3ecd and 48 bp dsRNA form a 2:1 complex: As described in the main text, gel filtration experiments suggested that 48 bp dsRNA and TLR3ecd form a high-affinity complex with 2:1 TLR3ecd:dsRNA stoichiometry. In order to precisely measure the mass of the complex and confirm the stoichiometry, sedimentation equilibrium experiments were carried out at several loading ratios of TLR3ecd:dsRNA. At a 2:1 loading ratio, a single species was observed with a buoyant molecular mass of 64.8 ± 1.1 kDa, with excellent fits (Fig. A1.3). This value is consistent with 2:1 stoichiometry, based on the buoyant mass of each pure species, and it corresponds to an experimental molecular mass of 219 ± 4 kDa and a partial specific volume of 0.698 cm^3g^{-1}. The absence of free dsRNA or TLR3ecd at this ratio suggests a high affinity interaction. To demonstrate that no higher-order complexes were formed when TLR3ecd was in excess, we next used a 4:1 loading ratio. These data fit poorly to a single species model, but excellent fits were observed when data were modeled as two non-interacting species corresponding to the 2:1 complex and excess TLR3ecd (Fig. A1.4). Fixing the buoyant mass of the smallest species to that of free TLR3ecd, a global data analysis returns a buoyant molecular mass of 68.9 ± 2.3 kDa for the complex, which corresponds to an experimental molecular mass of 232 ± 8 kDa. Similarly, when a loading ratio of 1:1 was used, dsRNA was found to be in excess. Data fit poorly to a single species model, but excellent fits were returned when data were modeled as two non-interacting species (Fig. A1.5). Fixing the buoyant mass of the smaller species to that of the dsRNA, the complex was determined to have a buoyant molecular mass of 66.5 ± 1.6 kDa and an experimental molecular mass of 225 ± 5 kDa. These results confirm that the mass of the complex is consistent with a 2:1 TLR3ecd:dsRNA stoichiometry.

To look for evidence of low-affinity 1:1 TLR3ecd:dsRNA complexes (predicted to form at low frequency as precursors of the stable 2:1 complexes), the sedimentation velocity experiments described in Figure 3D of the main text were analyzed quantitatively (Table A1.1). The vast majority of the c(s) distributions could be attributed to a complex of ~200 kDa (based on the partial specific volume measured above) and either excess dsRNA or TLR3ecd, according to the loading ratio. However, at a loading ratio of 3:4, ~4% of the loaded dsRNA appeared in a shoulder at ~7.7 S, which might correspond to a low-prevalence 1:1 complex. In any case, the relative scarcity of this species compared to the abundance of the 2:1 complex further supports the conclusion that TLR3 binding to dsRNA is highly cooperative.

Table A1.1 Quantification of sedimentation velocity profile c(s) distributions.

TLR3ecd:dsRNA loading ratio	Loading conc. of TLR3ecd, dsRNA (μM)	Conc. of free dsRNA (μM)	Conc. of free TLR3ecd (μM)	Conc. of 2:1 complex (μM)
3:4	0.70, 0.93	0.56	-	0.34
3:2	1.60, 1.07	0.31	-	0.71
2:1	1.74, 0.87	0.10	-	0.73
3:1	2.49, 0.83	-	0.75	0.82

Calculated concentrations of free dsRNA, free TLR3ecd, and the complex are based on an integration of the c(s) distributions shown in Figure 3D in the main text. Loading concentrations are based on the measured absorbance at 260 nm and the calculated extinction coefficients of the components.

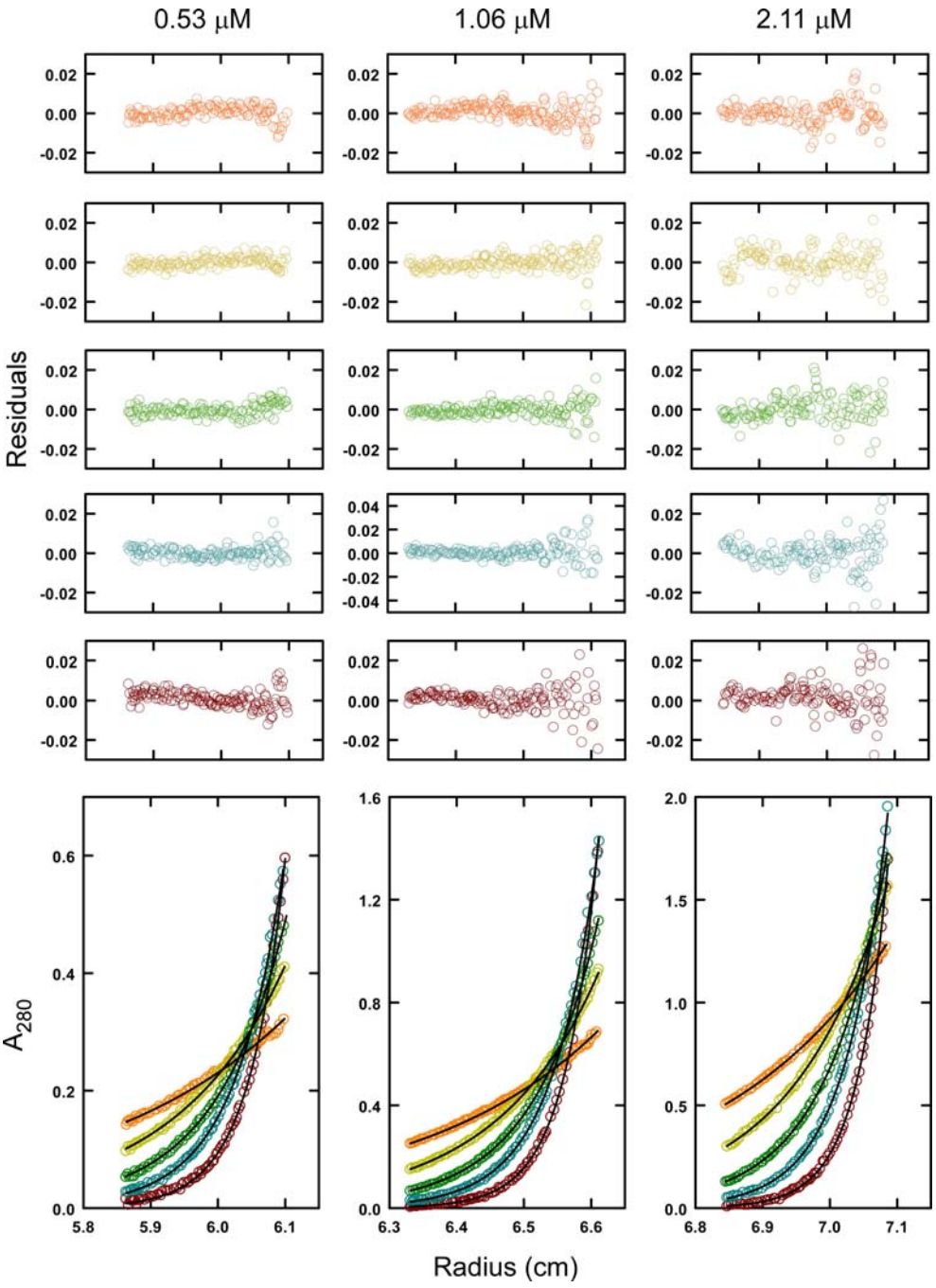


Figure A1.1 48 bp dsRNA is monodisperse and monomeric. Sedimentation equilibrium profiles obtained for dsRNA, at the indicated loading concentrations, collected at 9 (orange), 12 (yellow), 15 (green), 18 (cyan) and 21 (red) krpm. Data were analyzed globally in terms of a single ideal solute, with best fits to a molecular mass of 31.2 ± 0.25 kDa shown as black lines through the experimental points and corresponding residuals in upper panels.

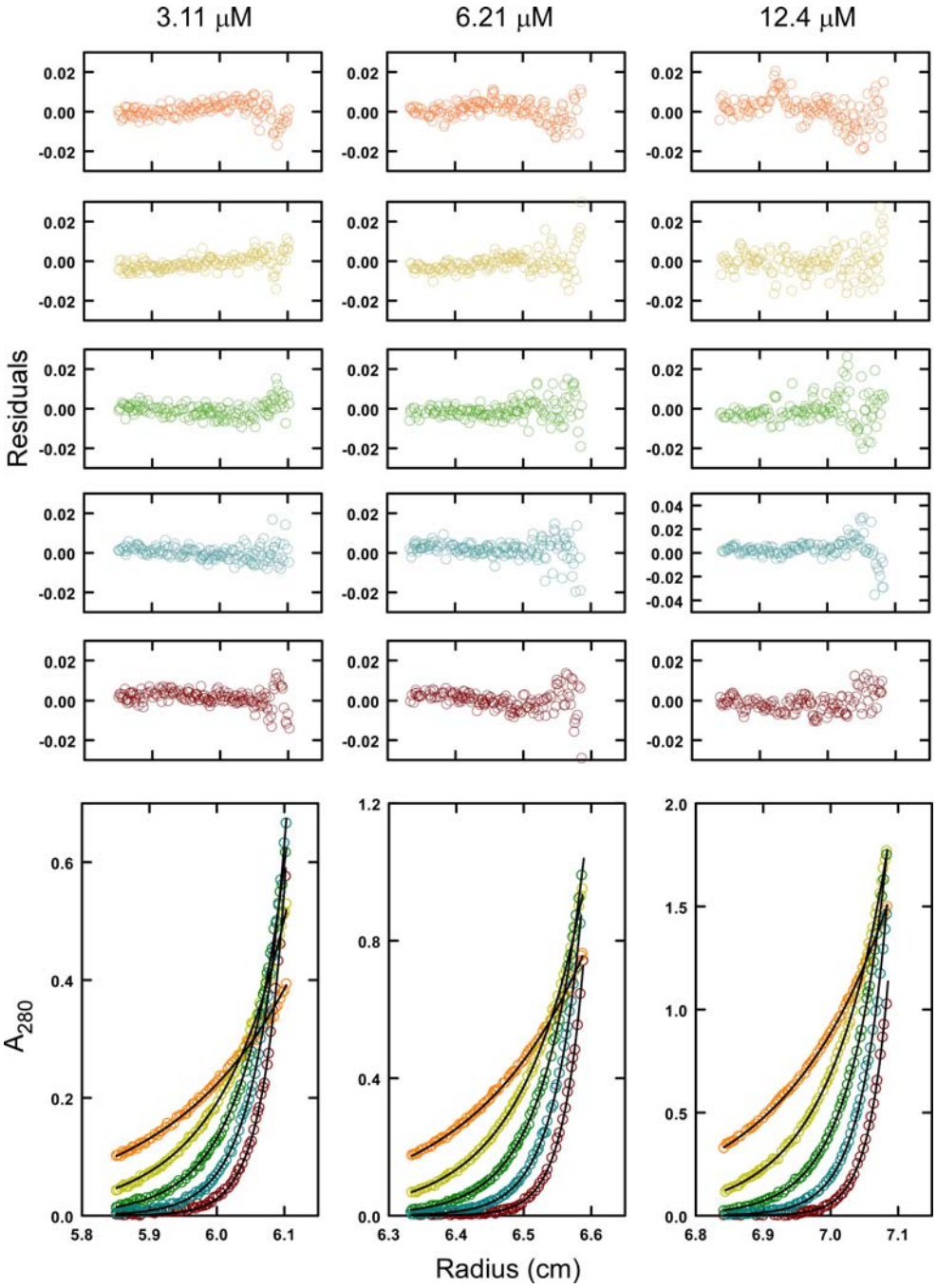


Figure A1.2 TLR3ecd is monodisperse and monomeric. Sedimentation equilibrium profiles obtained for the TLR3ecd, at the indicated loading concentrations, collected at 9 (orange), 12 (yellow), 15 (green), 18 (cyan) and 21 (red) krpm. Data were analyzed globally in terms of a single ideal solute, with best fits to a molecular mass of 93.0 ± 0.6 kDa shown as black lines through the experimental points and corresponding residuals in upper panels.

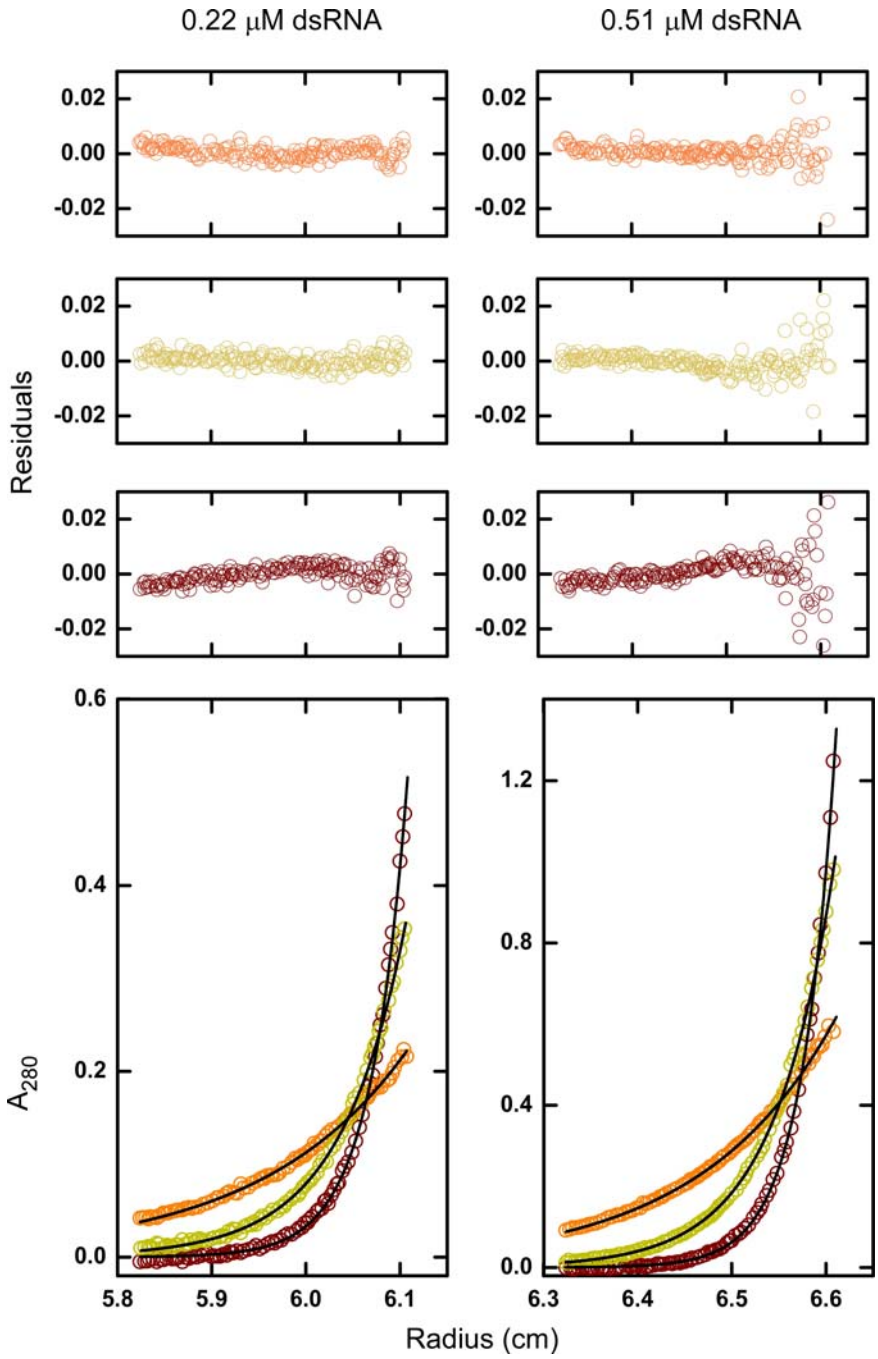
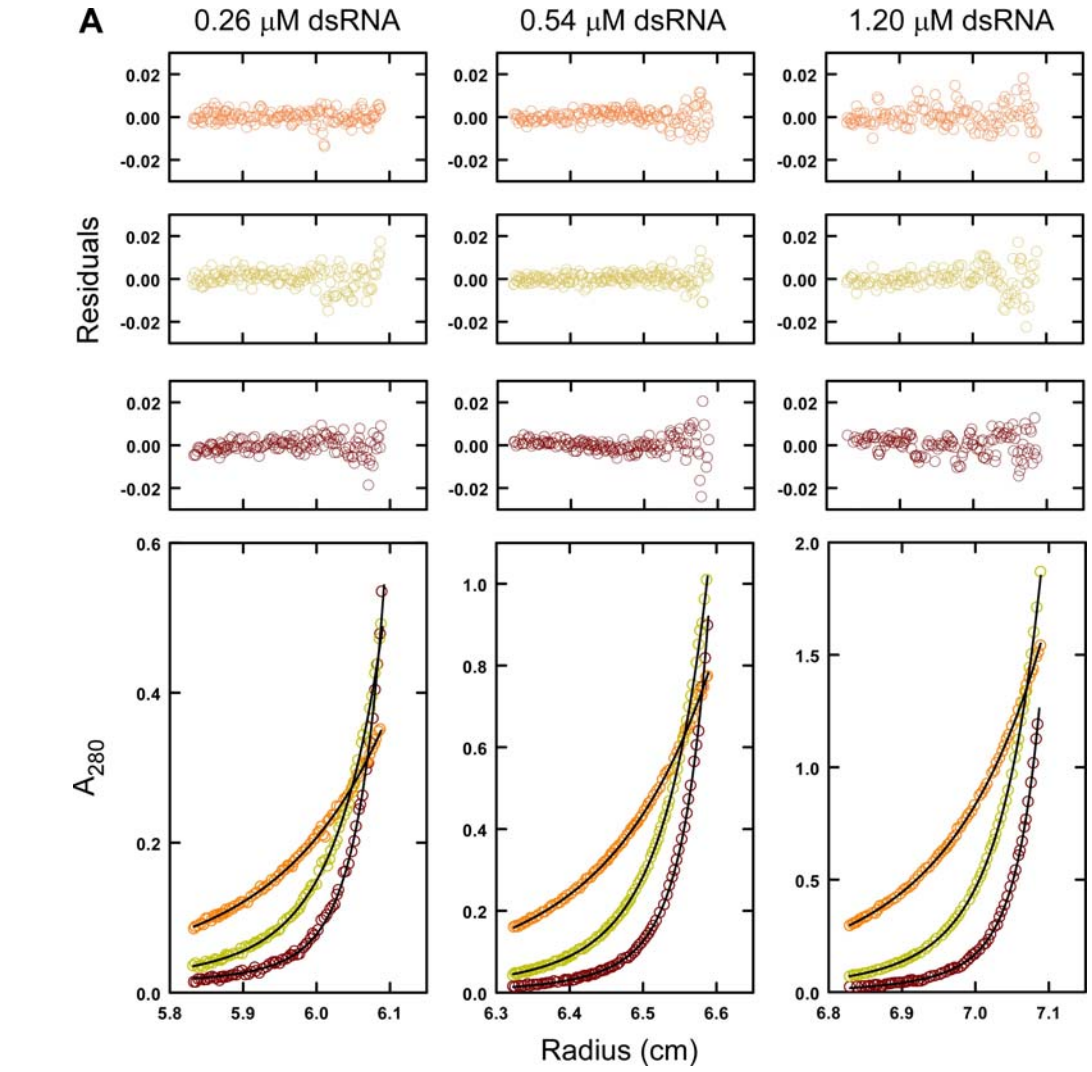


Figure A1.3 A 2:1 TLR3ecd:dsRNA mixture yields a single species. Sedimentation equilibrium profiles obtained for a 2:1 TLR3ecd:dsRNA mixture, at the indicated loading concentrations based on dsRNA, collected at 6 (orange), 9 (yellow) and 12 (red) krpm. Data were analyzed globally in terms of a single ideal solute, with best fits returning a molecular mass of 219 ± 4 kDa, as shown by black lines through the experimental points and corresponding residuals in upper panels.



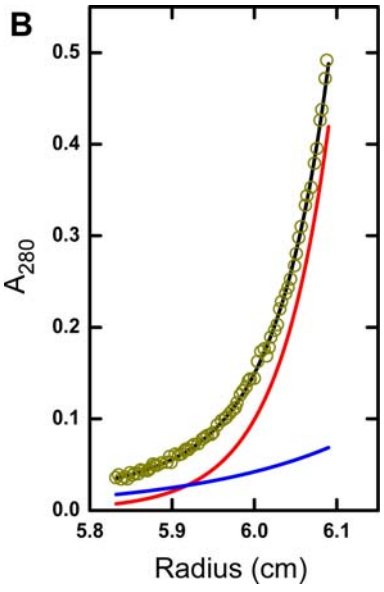


Figure A1.4 A 4:1 TLR3ecd:dsRNA mixture yields complex and excess TLR3ecd. (A) Sedimentation equilibrium profiles obtained for a 4:1 TLR3ecd:dsRNA mixture, at the indicated loading concentrations based on dsRNA, collected at 6 (orange), 9 (yellow) and 12 (red) krpm. Data were analyzed globally in terms of two non-interacting solutes, corresponding to free TLR3ecd and a complex. Best fits with a complex molecular mass of 232 ± 8 kDa are shown as black lines through the experimental points with the corresponding residuals shown above. (B) A representative overall profile can be decomposed into contributions from free TLR3ecd (blue) and the 2:1 TLR3ecd:dsRNA complex (red) (0.26 μM dsRNA, 9 krpm).

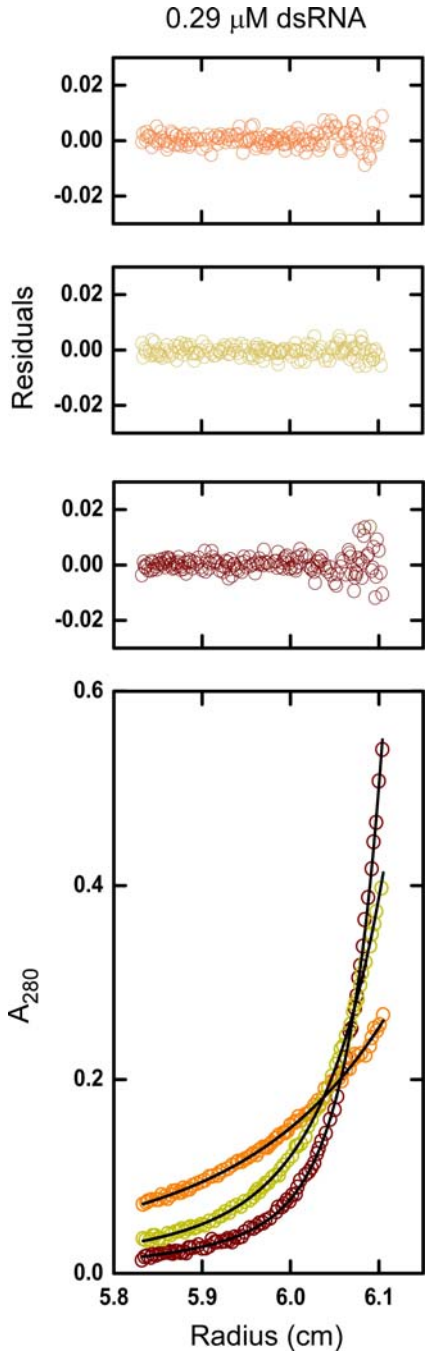


Figure A1.5 A 1:1 TLR3ecd:dsRNA mixture yields complex and excess dsRNA. Sedimentation equilibrium profiles obtained for a 1:1 TLR3ecd:dsRNA mixture, at a loading concentration of 0.29 μM based on dsRNA, collected at 6 (orange), 9 (yellow) and 12 (red) krpm. Data were analyzed in terms of two non-interacting solutes, corresponding to free dsRNA and a complex. Best fits with a complex molecular mass of 225 ± 5 kDa are shown as black lines through the experimental points, with the corresponding residuals shown above.

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