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Supporting information for article:

Solution structures of long-acting insulin analogues and their complexes with albumin

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S1. *Ab-initio* modelling results

S1.1. Detemir tri-hexamer

20 *ab-initio* models were generated by *DAMMIF* based on the *P(r)* function with a maximum intra-molecular dimension (D_{max}) of 11.3 nm. The curve has an ambiguity score of 1.56 which indicate that the *ab-initio* model might be ambiguous(Petoukhov & Svergun, 2015). The ensemble of models had a resolution of 42±3 Å and a normalized spatial discrepancy (*NSD*) of 0.67±0.02 indicating a stable solution. A cluster analysis was performed by *DAMCLUST*, resulting in three clusters. The most typical model of one of the clusters ($\chi^2 = 0.80$) is presented in Fig. 2*c* in a light blue surface representation. The model has a bent *v*-shape and seems to consist of three distinct spheres that could correspond to three hexamers.

S1.2. Degludec albumin-di-hexamer complex

20 *ab-initio* models were generated by *DAMMIF* based on the SEC-SAXS$_{\text{albumin-degludec}}$ *P(r)* function with a maximum intramolecular dimension (D_{max}) of 13.4 nm. The ambiguity score of the scattering curve was calculated by *AMBIMETER* to 1.40 indicating that a shape reconstruction is potentially unique. The ensemble of models had a resolution of 41±3 Å and an *NSD* of 0.71±0.04 indicating a stable solution. The most typical model of the ensemble ($\chi^2 = 1.67$) is presented in Fig. 7*c* (light blue surface representation).

S1.3. Detemir albumin-hexamer complex

20 *DAMMIF* models were generated based on the albumin-detemir 8.5 mg/mL *P(r)* function with a D_{max} of 13.0 nm and thereafter aligned and averaged by *DAMAVER* to an average model. The scattering curve has an ambiguity score of 1.230 indicating that unambiguous shape reconstruction should be possible.The ensemble of models had a resolution of 39±3 Å and an *NSD* of 0.68±0.02 indicating a stable solution, which is expected from the low ambiguity score. The most typical model of the ensemble ($\chi^2 = 0.86$) is presented in Figs. 9*c* and 9*d* from different orientations (light blue).

S1.4. Detemir albumin-di-hexamer-albumin complex

Ab-initio modelling was carried out applying P1 and P2 symmetry based on the albumin-detemir 15.6 mg/mL *P(r)* function with D_{max} of 19.5 nm. *DAMMIF* was run 20 times to generate models with P1 symmetry, and *DAMMIN* was run 20 times to generate models with P2 symmetry. The scattering curve has an ambiguity score of 2.076 indicating that shape reconstruction might be ambiguous.

The ensemble of models with P1 symmetry had a resolution of 53±4 Å and with *NSD* of 1.0±0.1. The ensemble of models with P2 symmetry had a resolution was 55±4 Å and an NSD 0.76±0.08. The most typical models of the ensembles with respectively P1 ($\chi^2 = 0.83$) and P2 symmetry ($\chi^2 = 0.89$) are

presented in Figs. 10*c* and 10*d*. Although a stable solution was not obtained for the models with P1 symmetry (*NSD* > 0.7(Volkov & Svergun, 2003)), there is a good agreement between the two representative models that are both elongated.

Table S1 Sample overview

Overview over samples listing their constituents, buffer, total protein concentration, molar ratio, and the beamline used for data collection.

Insulin	Buffer	Protein conc. (mg/mL)	albumin:insulin	Data collection
Detemir	Buf_{det}	0.5-9.9	-	I911-SAXS (MAXII, MAXIVLab)
Degludec	Buf_{deg}	0.5-7.7	-	P12 (PETRAIII, DESY)
Detemir	$Buf_{alb\text{-}det}$	1.9-20.8	1:6	I911-SAXS (MAXII, MAXIVLab)
Degludec	Buf_{deg}	10.6 (SEC-SAXS)	1:6	P12 (PETRAIII, DESY)
Degludec	Buf_{deg}	1.5-15.3	1:6	P12 (PETRAIII, DESY)
Degludec	Buf_{deg}	2.1-10.8	1:12	P12 (PETRAIII, DESY)

Table S2 Degludec samples – molecular parameters

Molecular parameters for degludec samples derived from SAXS analysis. The subscripts indicate whether the parameter is derived from Guinier analysis (G) or the $P(r)$ function (P). $\#_{monomers}$ denotes the average number of monomers calculated from MM^{P}.

C	$(I(0)/C)^{G}$	$(I(0)/C)^{P}$	R_g^{G}	R_g^{P}	D_{max}	V	MM^{G}	MM^{P}	MM^{V}	$\#_{monomers}$
mg/mL	mL/mg	mL/mg	nm	nm	nm	nm^3	kDa	kDa	kDa	
0.5	0.055	0.057	2.61	2.65	8.14	108.03	75.8	78.4	72.0	13.3
1.2	0.054	0.052	2.60	2.63	7.93	108.43	75.2	71.6	72.3	12.1
1.6	0.053	0.055	2.55	2.60	7.51	111.59	73.8	76.4	74.4	12.9
2.7	0.051	0.055	2.51	2.56	7.42	109.52	70.6	75.6	73.0	12.8
3.9	0.051	0.051	2.43	2.56	7.42	109.53	71.0	71.0	73.0	12.0
5.7	0.049	0.052	2.34	2.57	7.49	102.72	67.6	72.4	68.5	12.3
7.7	0.048	0.051	2.29	2.56	7.56	99.90	65.2	70.6	66.6	12.0

Table S3 Detemir samples – molecular parameters

Molecular parameters for detemir samples derived from SAXS analysis. The subscripts indicate whether the parameter is derived from Guinier analysis (G) or the $P(r)$ function (P). $\#_{monomers}$ denotes the average number of monomers calculated from MM^{P}.

C	$(I(0)/C)^{G}$	$(I(0)/C)^{P}$	R_g^{G}	R_g^{P}	D_{max}	V	MM^{G}	MM^{P}	MM^{V}	$\#_{monomers}$
mg/mL	mL/mg	mL/mg	nm	nm	nm	nm^3	kDa	kDa	kDa	
0.5	0.069	0.076	3.31	3.34	11.50	124.16	95.1	105.7	82.8	17.9
1.0	0.069	0.070	3.29	3.35	11.35	133.81	96.1	97.5	89.2	16.5
2.5	0.072	0.072	3.26	3.32	11.16	137.24	100.3	100.3	91.5	17.0
5.0	0.074	0.076	3.07	3.34	10.45	151.90	103.0	105.8	101.3	17.9
9.9	0.086	0.092	3.29	3.53	11.15	183.64	119.8	128.1	122.4	21.7

Table S4 Albumin-degludec (1:12) samples – molecular parameters

Molecular parameters for albumin-degludec samples in a 1:12 ratio derived from SAXS analysis. The subscripts indicate whether the parameter is derived from Guinier analysis (G) or the $P(r)$ function (P). $\#_{Alb\text{-}hex}$ and $\#_{Alb\text{-}di\text{-}hex}$ denote the average numbers of respectively albumin-hexamer and albumin-di-hexamer complex calculated from MM^{P}.

C	$(I(0)/C)^{G}$	$(I(0)/C)^{P}$	R_g^{G}	R_g^{P}	D_{max}	V	MM^{G}	MM^{P}	MM^{V}	$\#_{Alb\text{-}hex}$	$\#_{Alb\text{-}di\text{-}hex}$
mg/mL	mL/mg	mL/mg	nm	nm	nm	nm^3	kDa	kDa	kDa		
2.1	0.010	0.010	3.77	3.84	13.61	181.10	138.5	138.5	120.7	1.3	1.0
4.4	0.010	0.010	3.77	3.86	13.56	189.10	137.9	137.9	126.1	1.3	1.0
6.5	0.010	0.010	3.73	3.81	13.12	189.28	140.6	140.6	126.2	1.4	1.0
10.8	0.010	0.010	3.6	3.73	12.22	185.11	137.0	138.2	123.4	1.3	1.0

Table S5 Albumin-degludec (1:6) samples – molecular parameters

Molecular parameters for albumin-degludec samples in a 1:6 ratio derived from SAXS analysis. The subscripts indicate whether the parameter is derived from Guinier analysis (G) or the $P(r)$ function (P). $\#_{\text{Alb-hex}}$ and $\#_{\text{Alb-di-hex}}$ denote the average numbers of respectively albumin-hexamer and albumin-di-hexamer complex calculated from MM^{P}.

C	$(I(0)/C)^{G}$	$(I(0)/C)^{P}$	R_g^{G}	R_g^{P}	D_{max}	V	MM^{G}	MM^{P}	MM^{V}	$\#_{\text{Alb-hex}}$	$\#_{\text{Alb-di-hex}}$
mg/mL	mL/mg	mL/mg	nm	nm	nm	nm^3	kDa	kDa	kDa		
1.5	0.010	0.010	3.99	4.11	14.81	208.91	144.8	144.8	139.3	1.4	1.0
3.1	0.011	0.011	4.04	4.14	14.44	222.00	153.4	157.9	148.0	1.5	1.1
4.7	0.011	0.011	4.04	4.16	14.7	226.58	156.5	156.5	151.1	1.5	1.1
7.6	0.012	0.012	3.93	4.09	13.56	225.21	159.4	163.0	150.1	1.6	1.2
10.2	0.011	0.011	3.85	4.04	13.37	221.94	153.6	153.6	148.0	1.5	1.1
15.13	0.011	0.011	3.53	3.93	12.67	214.82	146.5	152.0	143.2	1.5	1.1

Table S6 Albumin-degludec (1:6) SEC-SAXS sample – molecular parameters

Molecular parameters for albumin-degludec SEC-SAXS sample in a 1:6 ratio derived from SAXS analysis. The subscripts indicate whether the parameter is derived from Guinier analysis (G) or the $P(r)$ function (P). $\#_{\text{Alb-hex}}$ and $\#_{\text{Alb-di-hex}}$ denote the average numbers of respectively albumin-hexamer and albumin-di-hexamer complex calculated from MM^{P}.

Elution	C	$(I(0)/C)^{G}$	$(I(0)/C)^{P}$	R_g^{G}	R_g^{P}	D_{max}	V	MM^{G}	MM^{P}	MM^{V}	$\#_{\text{Alb-hex}}$	$\#_{\text{Alb-di-hex}}$
Frame	mg/mL	mL/mg	mL/mg	nm	nm	nm	nm^3	kDa	kDa	kDa		
1124	1.8	0.056	0.060	2.97	3.06	9.34	117.71	77.6	83.1	78.5	0.8	0.6
994	6.5	0.102	0.102	3.75	3.77	13.40	179.46	141.3	141.3	119.6	1.3	1.0

Table S7 Albumin-detemir (1:6) samples – molecular parameters

Molecular parameters for albumin-detemir samples in a 1:6 ratio derived from SAXS analysis. The subscripts indicate whether the parameter is derived from Guinier analysis (G) or the $P(r)$ function (P). $\#_{\text{Alb-hex}}$ and $\#_{\text{Alb-di-hex-alb}}$ denote the average numbers of respectively albumin-hexamer and albumin-di-hexamer-albumin complex calculated from MM^{P}.

C	$(I(0)/C)^{\text{G}}$	$(I(0)/C)^{\text{P}}$	R_g^{G}	R_g^{P}	D_{max}	V	MM^{G}	MM^{P}	MM^{V}	$\#_{\text{Alb-hex}}$	$\#_{\text{Alb-di-hex-alb}}$
mg/mL	mL/mg	mL/mg	nm	nm	nm	nm^3	kDa	kDa	kDa		
1.9	0.057	0.057	3.14	3.20	10.82	125.03	78.3	78.3	83.4	0.8	0.4
4.1	0.054	0.054	3.08	3.15	10.71	126.58	74.6	74.6	84.4	0.7	0.4
8.5	0.074	0.075	3.54	3.64	13.03	162.34	102.1	103.7	108.2	1.0	0.5
15.6	0.150	0.152	5.41	5.69	19.5	309.45	208.3	211.0	206.3	2.1	1.0
20.8	0.275	0.399	7.79	8.31	27.25	650.76	380.9	552.4	433.8	5.4	2.7

Table S8 SAS experimental details

This table has been prepared according to guidelines published by Trewhella *et al.* (2017), Acta Cryst. D73, 710-728, https://doi.org/10.1107/S2059798317011597.

(*a*) Sample details						
	Detemir tri-hexamer	1:6 complex, FA4	1:6 complex, FA7	2:12 complex, P1	2:12 complex, P2	1:12 complex
Organism	*Homo sapiens*	*Homo sapiens*	*Homo sapiens*	*Homo sapiens*	*Homo sapiens*	*Homo sapiens*
Source	Levemir®, Novo Nordisk A/S	Recombumin® Alpha, Albumedix Ltd., Levemir®, Novo Nordisk A/S.				Recombumin® Elite, Albumedix Ltd., Tresiba®, Novo Nordisk A/S.
Uniprot sequence ID (residues in construct), modifications	Insulin detemir: P01308 (25-53, 90-110), lipidated with myristic acid at Lys^{B29}	Insulin detemir: P01308 (25-53, 90-110), lipidated with myristic acid at Lys^{B29} and human serum albumin: P02768 (25-609)				Insulin degludec: P01308 (25-53, 90-110), lipidated with hexadecanedioic acid at Lys^{B29} through γ-glutamate linker and human serum albumin: P02768 (25-609)
Extinction coefficient (A_{280}, $M^{-1}cm^{-1}$)	Albumin: 34445 [1]					
Partial specific volume $\bar{\upsilon}$ ($cm^3\,g^{-1}$)	0.7425					
Particle contrast $\Delta\bar{\rho}$ (10^{10} cm^{-2})	2.09					
Molecular mass *M* from chemical	Detemir: 5.9	Detemir: 5.9, albumin: 66.5				Degludec: 6.1, albumin: 66.5

[1] Not used for detemir or degludec as they are in phenol-containing buffers

composition (kDa)						
Expected molecular mass of complex M_E (kDa)	106.2	101.9	101.9	203.8	203.8	139.7
For SEC-SAS, loading concentration, (mg ml^{-1}), injection volume (µl), flow rate (ml min^{-1})						10.6, 100, 0.70
Concentration (mg ml^{-1}) measured and method	2.5, SAXS scaling	8.5, SAXS scaling	8.5, SAXS scaling	15.6, SAXS scaling	15.6, SAXS scaling	6.5, SAXS scaling
Solvent composition	5.0 mM Na_2HPO_4, 13.1 mM *m*-cresol, 15.1 mM phenol, 173.7 mM glycerol, 20.0 mM NaCl	6.9 mM Na_2HPO_4, 11.9 mM *m*-cresol, 13.7 mM phenol, 157.3 mM glycerol, 38.5 mM NaCl		8.8 mM Na_2HPO_4, 10.6 mM *m*-cresol, 12.2 mM phenol, 140.9 mM glycerol, 56.9 mM NaCl		25 mM Na_2HPO_4, 15.9 mM *m*-cresol, 15.9 mM phenol, 212.8 mM glycerol, 20 mM NaCl
(*b*) SAS data collection parameters						
Source, instrument and description or reference	I911-SAXS beamline(Labrador *et al.*, 2013), MAXII, MAX IV Laboratory					P12 BioSAXS beamline(Blanchet *et al.*, 2015), PETRAIII
Wavelength (Å)	0. 9100					1.241
Beam size (µm)	300 × 200					200 × 120

Sample-to-detector distance) (mm)	1962	3000
q-measurement range (nm^{-1})	0.0829-5.406	0.0248-5.036
Absolute scaling method	Comparison with scattering from pure H_2O	
Basis for normalization to constant counts	To transmitted intensity by beam-stop counter	
Method for monitoring radiation damage	Frame-by-frame comparison	
Exposure time	4 × 30	Continuous 1 s data-frame measurements of SEC elution
Sample configuration including path length and flow rate where relevant	Flow cell, effective sample path length 1.5 mm.	SEC–SAXS with flow cell, effective sample path length 1.7 mm
Sample temperature (ºC)	20	20

(*c*) Software employed for SAS data reduction, analysis and interpretation	
SAS data reduction to sample–solvent scattering, and extrapolation, merging, desmearing.	The *PyFAI*(Kieffer & Wright, 2013) package, *PRIMUSqt*(Konarev *et al.*, 2003) and *CHROMIXS*(Franke *et al.*, 2017) from *ATSAS* 2.8.3(Franke *et al.*, 2017)
Calculation of ε from sequence	*ProtParam*(Gasteiger *et al.*, 2005) tool from *ExPaSy*(Gasteiger *et al.*, 2003)

Basic analyses: Guinier, $P(r)$, V_P	*PRIMUSqt*(Konarev *et al.*, 2003) from *ATSAS* 2.8.3(Franke *et al.*, 2017)
Shape/bead modelling	*DAMMIF*(Franke & Svergun, 2009), *DAMMIN*(Svergun, 1999), *DAMAVER*(Volkov & Svergun, 2003) and DAMCLUST(Petoukhov *et al.*, 2012) from *ATSAS* 2.8.3(Franke *et al.*, 2017)
Atomic structure modelling	*SASREFCV*(Petoukhov & Svergun, 2006, 2005) and *CRYSOL*(Svergun *et al.*, 1995) from *ATSAS* 2.8.3(Franke *et al.*, 2017)
Molecular graphics	*PyMOL* (version 1.8.2.3, Schrödinger, LLC)

(*d*) Structural parameters

	Detemir tri-hexamer	1:6 complex, FA4	1:6 complex, FA7	2:12 complex, P1	2:12 complex, P2	1:12 complex
Guinier Analysis						
$I(0)$ (cm^{-1})	0.18	0.63	0.63	2.35	2.35	0.66
R_g (Å)	32.6	35.4	35.4	54.1	54.1	38
q-range ($Å^{-1}$)	0.013-0.038	0.012-0.037	0.012-0.037	0.016-0.024	0.016-0.024	0.008-0.034
Quality	94 %	96 %	96 %	80 %	80 %	98 %
M from $I(0)$ (ratio to expected value)	100.3 (0.94)	102.1 (1.00)	102.1 (1.00)	208.3 (1.02)	208.3 (1.02)	138.5 (0.99)
$P(r)$ analysis						
$I(0)$ (cm^{-1})	0.18	0.64	0.64	2.38	2.38	0.66
R_g (Å)	33.2	36.4	36.4	56.9	56.9	37.7
D_{max} (Å)	111.6	130.3	130.3	195.0	195.0	134.0
q-range ($Å^{-1}$)	0.013-0.245	0.012-0.226	0.012-0.226	0.016-0.147	0.016-0.147	0.008-0.213
Total quality estimate	0.70	0.67	0.67	0.53	0.53	0.81
M from $I(0)$ (ratio to expected value)	100.3 (0.94)	103.7 (1.02)	103.7 (1.02)	211.0 (1.04)	211.0 (1.04)	138.5 (0.99)

Porod volume, V_P (nm^3)	137.20	162.34	162.34	309.45	309.45	179.50
Ratio V_P/calculated M	1.37	1.57	1.57	1.46	1.46	1.30
(*e*) Shape modelling results						
	Detemir tri-hexamer	1:6 complex, FA4	1:6 complex, FA7	2:12 complex, P1	2:12 complex, P2	1:12 complex
DAMMIF (Run in interactive mode with dummy atom radius 2.7 Å, otherwise default parameters, 20 calculations)						
q-range for fitting ($Å^{-1}$)	0.013-0.245	0.012-0.225	0.012-0.225	0.016-0.147		0.008-0.213
Shape	compact	compact	compact	compact		
Symmetry, anisotropy assumptions	P1, none	P1, none	P1, none	P1, none		P1, none
NSD (standard deviation), No. of clusters	0.67 (0.02), 3	0.68 (0.02), 7	0.68 (0.02), 7	0.95 (0.11), 4		0.71 (0.04), 4
χ^2 range	0.785-0.862	0.794-0.924	0.794-0.924	0.802-0.874		1.619-1.701
Constant adjustment to intensities	-	1.19×10^{-4}	1.19×10^{-4}	-		3.33×10^{-5}
M estimate (kDa)	78.5	95.7	95.7	199		105
Resolution (from *SASRES*) (Å)	42 (3)	39 (3)	39 (3)	53 (4)		41 (3)
DAMMIN (Run in expert mode with default parameters, 20 calculations)						
q-range for fitting					0.016-0.147	
Initial search volume					ellipsoid	

Symmetry, anisotropy assumptions	P2, prolate anisometry (perpendicular symmetry and anisometry axes)
NSD (standard deviation), No. of clusters	0.76 (0.08), 4
χ^2range	0.864-0.915
Constant adjustment to intensities	1.59×10^{-4}
Resolution (from *SASRES*) (Å)	55 (4)

(*f*) Atomistic modelling

	Detemir tri-hexamer	1:6 complex, FA4	1:6 complex, FA7	2:12 complex, P1	2:12 complex, P2	1:12 complex
SASREF (default parameters, 10 calculations, data shown for best model)						
q-range for fitting ($Å^{-1}$)	0.008-0.415	0.008-0.432	0.008-0.432	0.008-0.432	0.008-0.432	0.005-0.403
Symmetry	P1	P1	P1	P1	P2	P1
χ^2 value	0.91	1.68	0.93	0.92	0.95	2.62
Constant adjustment to intensities	8.87×10^{-5}	3.77×10^{-5}	4.31×10^{-5}	6.11×10^{-5}	6.62×10^{-5}	8.77×10^{-5}
CRYSOL						
q-range for fitting	0.008-0.400	0.008-0.400	0.008-0.400	0.008-0.400	0.008-0.400	0.005-0.400
No constant subtraction						
χ^2 value	1.16	1.88	1.32	1.07	1.21	2.57
Predicted R_g (Å)	33.20	34.83	35.35	56.30	56.99	37.61

Vol (Å), Ra (Å), Dro (e Å^{-3})	127959, 1.40, 0.030	131398, 1.40, 0.020	131398, 1.48, 0.020	265264, 1.80, 0.025	265264, 1.80, 0.025	175546, 1.80, 0.018
Constant subtraction allowed						
χ^2 value	1.54, 0.00	1.88	1.30	1.01	1.12	1.74,
Predicted R_g (Å)	33.11	34.83	35.35	56.38	57.05	37.68
Vol (Å), Ra (Å), Dro (e Å^{-3})	136194, 1.76, 0.022	131398, 1.40, 0.020	130781, 1.40, 0.020	260328, 1.50, 0.028	231562, 1.40, 0.025	163125, 1.40, 0.022
(*g*) SASBDB IDs for data and models						
	Detemir tri-hexamer SASDEV5	1:6 complex, FA4 SASDEW5	1:6 complex, FA7 SASDEX5	2:12 complex, P1 SASDEY5	2:12 complex, P2 SASDEZ5	1:12 complex SASDE26

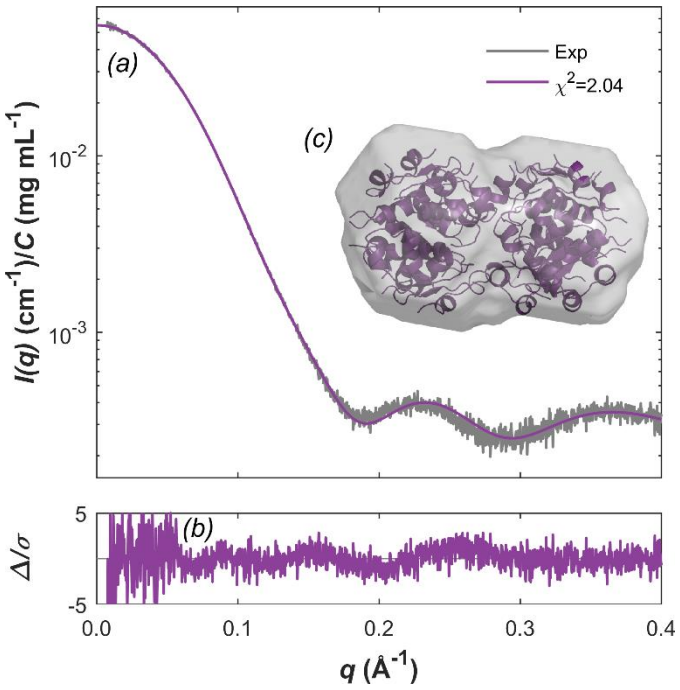

Figure S1 Structure of degludec R_3T_3-T_3R_3 di-hexamer with an inter-hexamer distance of 35.4 Å. (*a*) Fit of the structure (purple) to experimental degludec data extrapolated to infinite dilution (grey). The lower inset (*b*) shows error-weighted residual plots for the models. (*c*) The di-hexamer structure (purple) is superimposed onto the low resolution *ab-initio* model (grey).

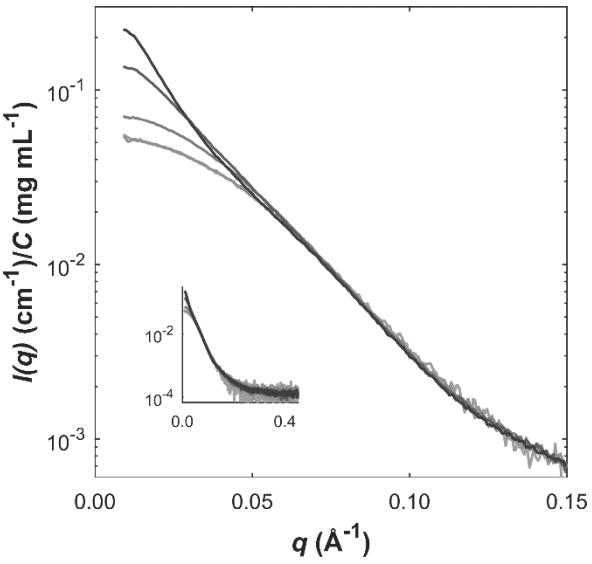

Figure S2 Scattering curves normalized for concentration of albumin-detemir mixed in a 1:6 ratio. The total protein concentration range is between 1.9-20.8 mg/mL with darker shades corresponding to higher concentrations. An increase in *I(q)/C* is observed corresponding to a concentration-dependent equilibrium.